# BIOLOGICAL BULLETIN

OF THE

## Marine Biological Laboratory

WOODS HOLE, MASS.


### Editorial Staff

GARY N. CALKINS—*Columbia University.*

E. G. CONKLIN—*Princeton University.*

M. H. JACOBS—*University of Pennsylvania.*

FRANK R. LILLIE—*University of Chicago.*

GEORGE T. MOORE—*The Missouri Botanic Garden.*

T. H. MORGAN—*Columbia University.*

W. M. WHEELER—*Harvard University.*

E. B. WILSON—*Columbia University.*

### Managing Editor

C. R. MOORE—*The University of Chicago.*



VOLUME LVI.

WOODS HOLE, MASS.

JANUARY TO JUNE, 1929

LANCASTER PRESS, INC.
LANCASTER, PA

Science Lib
Walter

# Contents of Volume LVI

No. 1. January, 1929.

Turner, C. L. *Studies on the Secondary Sexual Characters of Crayfishes, IX. Females of Cambarus with Aberrant Female Characters* ..... 1

MacKay, Margaret E. *The Digestive System of the Eel-Pout (Zoarces Anguillaris)* ..... 8

MacKay, Margaret E. *Note on the Bile in Different Fishes* 24

Richards, Oscar W. *The Effect of Neurophil Drugs on the Fiddler Crab, Uca pugnax* ..... 28

Richards, Oscar W. *The Conduction of the Nervous Impulse through the Pedal Ganglion of Mytilus* ..... 32

Crabb, Edward D. *Growth of a Pond Snail, Lymnæa stagnalis appressa, as Indicated by Increase in Shell Size* 41

Morita, Yun-Ichi, and Chambers, Robert. *Permeability Differences between Nuclear and Cytoplasmic Surfaces in Amœba dubia* ..... 64

Hopkins, D. L., and Johnson, Percy L. *The Culture of Amœba proteus in a Known Salt Solution* ..... 68

No. 2. February, 1929.

Okada, Yô K. *Supplementary Note on the Double Formation in the Echinus Germ in Diluted Sea Water* ..... 75

Dawson, J. A., and Belkin, Morris. *The Digestion of Oils by Amœba proteus* ..... 80

Butcher, Earl O. *The Origin of the Germ Cells in the Lake Lamprey (Petromyzon marinus unicolor)* ..... 87

Fry, Henry J. *The So-called Central Bodies in Fertilized Echinarachnius Eggs* ..... 101

Nabours, Robert K., and Foster, Martha E. *Parthenogenesis and the Inheritance of Color Patterns in the Grouse Locust Paratettix texanus Hancock* ..... 129

No. 3. March, 1929.

THORNER, MELVIN W. *Recovery of the Heart Beat of Fundulus Embryos After Stoppage by Potassium Chloride* 157
REICHLE, HERBERT S. *The Diagnosis of the Type of Twinning* . . . . . 164
WHEDON, ARTHUR D. *Muscular Reorganization in the Odonata During Metamorphosis* . . . . . 177
SUMWALT, MARGARET. *Potential Differences Across the Chorion of the Fundulus Egg* . . . . . 193
GEROULD, JOHN H. *History of the Discovery of Periodic Reversal of Heart-Beat in Insects* . . . . . 215
LAMBERT, W. V., AND CURTIS, V. *Further Studies on the Sex Ratio in the Chicken* . . . . . 226

No. 4. April, 1929.

NABRIT, S. MILTON. *The Rôle of the Fin Rays in the Regeneration in the Tail-fins of Fishes* . . . . . 235
MOENCH, G. L., AND HOLT, HELEN. *Microdissection Studies on Human Spermatozoa* . . . . . 267
YOCHEM, DONALD E. *Spermatozoön Life in the Female Reproductive Tract of the Guinea Pig and Rat* . . . . . 274
RICHARDS, OSCAR W. *The Correlation of the Amount of Sunlight with the Division Rates of Ciliates* . . . . . 298
COE, WESLEY R. *The Excretory Organs of Terrestrial Nemerteans* . . . . . 306

No. 5. May, 1929.

REICHLE, HERBERT S. *The Diagnosis of Monoövular Twinning* . . . . . 313
NADLER, ERNEST J. *Notes on the Loss and Regeneration of the Pellicle in Blepharisma undulans* . . . . . 327
FEDERIGHI, H. *Rheotropism in Urosalpinx cinerea Say* . . . . 331
MCCONNELL, CARL H. *Experimental Observations upon the Endodermal Glands of Pelmatohydra oligactis* . . . . . 341
HUFF, CLAY G. *Ovulation Requirements of Culex pipiens Linn* . . . . . 347
ROMANOFF, ALEXIS L. *Study of the Physical Properties of the Hen's Eggshell in Relation to the Function of Shell-Secretory Glands* . . . . . 351

MARTIN, J. HOLMES. *Effect of Excessive Dosages of Thyroid on the Domestic Fowl* ..... 357
BRINLEY, FLOYD J. *The Effect of Ammonium Salts on Protoplasm of Amœba* ..... 371

No. 6. JUNE, 1929.

RIZZOLO, ATTILIO. *The Effect of Caffeine and Theine upon the Excitability of the Spinal Cord* ..... 379
RIZZOLO, ATTILIO. *A Study of Equilibrium in the Smooth Dogfish—Galeus canis (Mitchill)* ..... 383
WENRICH, D. H. *The Structure and Behavior of Actinobolus vorax n. sp. (Protozoa, Ciliata)* ..... 390
KOSTOFF, DONTCHO, AND KENDALL, JAMES. *Studies on the Structure and Development of Certain Cynipid Galls* .... 402
DOMM, L. V. *The Effects of Bilateral Ovariotomy in the Brown Leghorn Fowl* ..... 459

SCIENCE LIBRARY

97

FEB 28 1929

# BIOLOGICAL BULLETIN

OF THE

**Marine Biological Laboratory**

WOODS HOLE, MASS.

VOL. LVI JANUARY, 1929 NO. 1

## CONTENTS

TURNER, C. L. *Studies on the Secondary Sexual Characters of Crayfishes, IX. Females of Cambarus with Aberrant Female Characters* ..... 1

MACKAY, MARGARET E. *The Digestive System of the Eel-Pout (Zoarces Anguillaris)* ..... 8

MACKAY, MARGARET E. *Note on the Bile in Different Fishes* ..... 24

RICHARDS, OSCAR W. *The Effect of Neurophil Drugs on the Fiddler Crab, Uca pugnax* ..... 28

RICHARDS, OSCAR W. *The Conduction of the Nervous Impulse through the Pedal Ganglion of Mytilus* .. 32

CRABB, EDWARD D. *Growth of a Pond Snail, Lymnæa stagnalis appressa, as Indicated by Increase in Shell Size* ..... 41

MORITA, YUN-ICHI, AND CHAMBERS, ROBERT. *Permeability Differences between Nuclear and Cytoplasmic Surfaces in Amœba dubia* ... 64

HOPKINS, D. L., AND JOHNSON, PERCY L. *The Culture of Amœba proteus in a Known Salt Solution* ..... 68

PUBLISHED MONTHLY BY THE

MARINE BIOLOGICAL LABORATORY

PRINTED AND ISSUED BY

LANCASTER PRESS, INC.

LANCASTER, PA.

AGENT FOR GREAT BRITAIN

WHELDON & WESLEY, LIMITED

*2, 3 and 4 Arthur Street, New Oxford Street, London, W. C. 2*

**Single Numbers, $1.00. Per Volume (6 numbers), $4.50**

Entered October 10, 1902, at Lancaster, Pa., as second-class matter under Act of Congress of July 16, 1894

## Editorial Staff

GARY N. CALKINS—*Columbia University.*
E. G. CONKLIN—*Princeton University.*
M. H. JACOBS—*University of Pennsylvania.*
FRANK R. LILLIE—*University of Chicago.*
GEORGE T. MOORE—*The Missouri Botanic Garden.*
T. H. MORGAN—*Columbia University.*
W. M. WHEELER—*Harvard University.*
E. B. WILSON—*Columbia University.*

---

## Managing Editor

C. R. MOORE—*The University of Chicago.*

---



All communications and manuscripts should be sent to the Managing Editor, the University of Chicago. Subscriptions and other matter should be addressed to the Biological Bulletin, Prince and Lemon Streets, Lancaster, Pa.

*Vol. LVI.* *January, 1929.* *No. 1.*

BIOLOGICAL BULLETIN

# STUDIES ON THE SECONDARY SEXUAL CHARACTERS OF CRAYFISHES.

## IX. Females of *Cambarus* with Aberrant Female Characters.

C. L. TURNER,

Zoölogical Laboratory, Northwestern University.

In the various genera of crayfishes the external apertures of the oviducts are placed at the bases of the third walking legs.

Desmarest in 1848 described a specimen of *Astacus fluviatilis* having supernumerary oviducal pores at the bases of the fourth walking legs. Upon dissection it was found that a normal pair of oviducts emerged from the sides of the ovary but that each oviduct bifurcated. The divided oviducts were attached at their distal ends to both normal and supernumerary apertures.

Benham, in 1891, described an unusual case of supernumerary oviducal pores in *Astacus fluviatilis*. Normal openings occurred at the bases of the third walking legs, but in addition to these a pair of oviducal pores appeared at the bases of the fifth walking legs. The oviducts divided after continuing for the greater part of their length as single tubes, one branch going to each oviducal opening.

Bateson, in 1894, after reviewing the described cases, offered fifteen new cases in *Astacus fluviatilis* in which females bore aberrant female characters. Four of these were specimens with only one oviducal pore at the base of a third walking leg instead of the usual pair. One of the specimens when dissected was found to have a normal oviduct on the side with the opening, but a blind and abortive tube on the other side. Seven specimens were described which had a single supernumerary pore on the right fourth leg. One was described in which supernumerary

pores were present in third, fourth, and fifth walking legs. In most of the cases the oviduct was divided on the side with the supernumerary pores, but in one or two cases the supernumerary pore occurred with no branch of the oviduct to connect with it. In the specimen having three pairs of oviducal pores, a pair of bifurcated oviducts extended to the pores on the third and fourth legs, but the pores on the fifth legs were blind.

Bateson was interested primarily in determining the proportion of specimens that were abnormal and in ascertaining whether the supernumerary pores formed a continuous or a discontinuous succession with the normal pores. His conclusions may be summarized as follows:

1. The cases with supernumerary pores are over 3 per cent. of the total number of *Astacus fluviatilis* examined.

2. The supernumerary pores are more likely to be unilateral than bilateral.

3. There is a clear succession between the legs with normal oviducal pores and those with supernumerary pores, the supernumerary pores always being smaller, although large enough in some cases to permit the passing of eggs.

4. The series may be an interrupted one, as shown by the case in which there are normal pores on the third legs and supernumerary pores on the fifth legs.

The matter of abnormal oviducal openings has been given little attention in the genus *Cambarus*. Undoubtedly cases like those to be described have been observed, but few if any have been described, and no attempt has been made to study the condition in *Cambarus* as Bateson has done in *Astacus fluviatilis*. The writer has been collecting and studying crayfishes with aberrant sexual characters for the past eight years, and among the thousands of specimens examined there have appeared twenty-one cases in females of *Cambarus virilis* and *Cambarus propinquus* which had aberrant conditions in the oviducal pores. In the region from which most of these specimens came (central and southeastern Wisconsin, northern Illinois, parts of Michigan, and northeastern Ohio) one of these two species has been the most abundant crayfish of the region, and attention has been confined almost entirely to these two. What the condition may

be in other species is not known, and not enough aberrant specimens have been collected to justify an opinion. The attempt has been made to study the relative proportion of aberrant and normal specimens in single localities in order to discover whether the aberrant cases are widely and uniformly distributed or whether they are concentrated in single localities.

## Description of Cases.

Case number one, a specimen of *Cambarus propinquus*, 52 mm. long, was collected from a small tributary of Turtle Creek at Carvers Rock, Wisconsin, on July 16, 1922. Normal genital apertures occur at the bases of the third walking legs, and in addition a fully developed genital aperture is found at the base of the left second walking leg. Upon dissection it appears that a normal oviduct occurs upon the right side but that two oviducts

Fig. 1

Fig. 2

occur on the left side (Fig. 2). The latter are joined together at the point of their union with the ovary and are attached to the oviducal pores at their distal ends. Figure one is a diagrammatic illustration of the relations of the ovary, oviducts, and oviducal pores in a normal female viewed from the ventral aspect. The Roman numerals indicate the walking legs.

Specimen number two, also one of *Cambarus propinquus*, 49 mm. long, was collected from South Kinnikinnic Creek, Illinois, a tributary of the Rock River, on June 30, 1924. Like specimen number one it is abnormal only in possessing an extra genital aperture. In this case the extra aperture is located at the base of the right second walking leg. The oviduct on the left side of the body is normal in every respect, but the one on the right side bifurcates at a point about one third of its length from the ovary, and the two branches become attached to the genital pores located in the second and third walking legs (Fig. 3).

A third abnormal specimen of *Cambarus propinquus*, 57 mm.

in length, was taken from Turtle Creek, Wisconsin, on July 5, 1924. A single normal oviducal pore is to be found on the left side of the body. There is no trace of the oviducal pore which is normally to be expected at the base of the third walking leg upon the right side of the body. The oviduct is entirely normal upon the left side, but a normal oviduct after leaving the ovary at the usual point and after proceeding for about four fifths of its normal length ends blindly, and the free end is attached to the exoskeleton of the leg by a few thin strands of connective tissue (Fig. 4).

Fig. 3

Fig. 4

Fig. 5

A fourth specimen of *Cambarus propinquus*, 42 mm. in length, from White River near Lake Geneva, Wisconsin, has a supernumerary oviducal pore at the base of the left second walking leg. The specimen was collected on July 2, 1924. Unfortunately the preservation is poor and the internal structure cannot be made out.

The fifth case was discovered in a collection from Pike River near Racine, Wisconsin. The specimen is 33 mm. in length and was taken on July 9, 1924. Beside the usual oviducal pores at the bases of the third walking legs, supernumerary oviducal pores are found on the bases of the second walking legs. The left oviduct is normal at its base but divides into two branches, the ends of which are attached to the normal and the supernumerary oviducal pores. On the right side there are two oviducts which arise from the ovary practically together and become attached at their distal ends to the normal and the supernumerary oviducal pores (Fig. 5).

A sixth specimen of *Cambarus propinquus*, 38 mm. in length, came from Turtle Creek near Beloit, Wisconsin, and was collected on July 5, 1925. The abnormality in this case consists of a well-developed supernumerary oviducal pore located on the base of the right second walking leg. Normal oviducts extend from

the ovary to the oviducal pores at the bases of the third legs, but the supernumerary oviducal pore is isolated with no duct of any kind attached to it (Fig. 6).

The seventh specimen has already been described in an earlier paper as a female bearing male secondary sex characters in the form of male first and second abdominal appendages and male genital pores at the bases of the fifth leg. In addition to the male characters, however, there is also a supernumerary female character in the form of a small oviducal pore upon the base of the left fourth walking leg. This supernumerary pore is much smaller than the normal one, but it is distinct and its character is unmistakable. In spite of the array of male and supernumerary female structures, the specimen proved to be a functional female, and was bearing embryos attached to the swimmerets when taken (May, 1923) (Fig. 7).

Fig. 6

Fig. 7

Three other cases were discovered in *Cambarus virilis* which were much like some of those described for *Cambarus propinquus*. One of them from a small tributary of the Wisconsin River near Middleton, Wisconsin, had supernumerary oviducal pores upon both second walking legs and the arrangement of the oviducts was much like that figured in Fig. 5. The other, collected on July 11, 1923, from the outlet of Muskego Lake, Wisconsin, had a supernumerary oviducal pore upon the right second walking leg but, the internal organs being in a very bad state of preservation, nothing could be made out as to the arrangement of the oviducts.

A collection of 356 female specimens of *Cambarus virilis* from the Wisconsin River between Rhinelander and Wisconsin Rapids, collected July, 1926, contained seven which had an unusual arrangement of the oviducal pores. Two had supernumerary pores upon the left second walking legs, two had supernumerary pores upon the right second legs, while the other three had supernumerary pores upon both the second walking legs.

A female specimen of *Cambarus virilis* from the Rock River (collected by David Thompson of the Illinois State Laboratory of Natural History, August, 1926) near Rockford, Illinois, had an oviducal pore only upon the left third walking leg.

A specimen of *Cambarus propinquus* collected in 1924 from School Section Creek near Grayling, Michigan, by Hankinson and Langlan had small supernumerary oviducal pores upon the second walking legs.

A medium-sized specimen of *Cambarus virilis* from Browns Lake, Michigan, collected July 2, 1907, by Baker, showed the usual oviducal pore upon the left third leg but failed to have one upon the right leg.

The last specimen was a small *Cambarus propinquus* female from Third Creek, Flint, Michigan (Kaufman collector) which had a full-sized oviducal pore upon the right second walking leg in addition to the usual pair upon the third legs.

## Discussion.

Bateson apparently did not study any considerable number of specimens of *Astacus* from a single locality to determine the proportion of aberrant to normal specimens for a single crayfish population. He leaves the impression, however, that a fair proportion of all the females are aberrant (over 3 per cent.). In *Cambarus* the proportion of aberrant to normal females is much less, at least in *Cambarus virilis* and *Cambarus propinquus*. Only an approximate record has been kept of the total number examined by the writer in these two species, but the total would be well over 15,000 females, and the twenty recorded here represent all of the females with unusual female characters. It should be emphasized that all were examined with attention focused upon the secondary sexual characters. Some facts are available as to whether these abnormalities represent a random scattering or a concentration in particular localities. In case number two, forty-seven females constituted the entire collection in which this one abnormality was found. Case four occurred in a collection of fifteen females, case three in a collection of 150 females, case five in a collection of 82 females, case eight in a collection of 110 females, case nine in a collection of 27 females,

case seventeen in a collection of 90 females, and in cases ten to sixteen a collection of 356 females yielded seven aberrant specimens. Except in the last instance, there does not seem to be any concentration in a particular locality. The data furnished in the descriptions makes it evident also that *Cambarus propinquus* is as likely to produce these aberrant females as *Cambarus virilis*.

When there are supernumerary oviducal pores in *Cambarus*, they are located usually upon the second walking legs. This is in marked contrast to the condition in *Astacus* where the supernumerary pores occur upon the fourth walking leg or more rarely upon the fifth. Apparently there are basic reasons for this persistent difference.

It should be noted in passing that true oviducal pores in *Cambarus* do not occur upon any other leg than the third unless the third also bears pores. When males of *Cambarus* bear oviducal pores, it is always upon the third leg also. It may be considered that, in *Astacus*, the third, fourth, and fifth walking legs are potential bearers of oviducal pores, and in *Cambarus* the second and third, but in both that the third leg is greatly emphasized in this respect.

In normal specimens a fully developed oviduct is attached to each oviducal pore. It is obvious that this relationship need not be definitely fixed and that they are not mutually interdependent in their embryology for guidance in direction of differentiation. If an oviduct required for its normal development that it be attached to an oviducal pore or a pore for its development upon an adjacent oviduct, then the cases represented in Figs. 4, 6, and 7 would be impossible. Here we have oviducal pores without oviducal tubes and tubes without pores.

## LITERATURE.

**Bateson, Wm.**
'94 Materials for the Study of Variation.
**Benham, W. B.**
'91 Ann. Mag. Nat. Hist., 1891, Series 6, VII., p. 256.
**Desmarest, E.**
'48 Ann. Soc. Ent. France, Series 2, VI., p. 479.

# THE DIGESTIVE SYSTEM OF THE EEL-POUT (*ZOARCES ANGUILLARIS*).

MARGARET E. MacKAY.

*(From the Atlantic Experimental Station, St. Andrews, N. B.)*

A systematic examination of the digestive system and its function in fishes, at different levels on the evolutionary scale, may greatly aid in the understanding of the digestive processes in higher animals, and in addition may shed some light on the relation between certain fish and other marine forms: a problem interesting from a theoretical as well as a practical point of view.

In the present study an attempt has been made to investigate the functions of the digestive system of the eel-pout (*Zoarces Anguillaris*) found in the Bay of Fundy. As no description of the alimentary tract and its appendages could be found in the literature available, a few macroscopical dissections were made in order to show the anatomical relations existing in the eel-pout. The drawings presented in this paper are all done from a single specimen, which measured 425 mms. from the snout to tip of the caudal fin.

The alimentary canal in the eel-pout is comparatively short and presents an œsophagus, stomach and intestine, of which the latter may be separated into a duodenum and small intestine. On opening the abdomen the ventral surfaces of the viscera are seen *in situ* (Fig. 1). The stomach, partially obscured by the liver, lies on the left side; during fasting this viscus is rather small, but in fed animals it may become tremendously distended. Pyloric cæca are absent, but there is a well-marked sphincter separating the stomach from the pear-shaped duodenum. The latter structure occupies a mid-ventral position from whence it curves posteriorly and to the right, gradually tapering into the small intestine. The intestine is bent upon itself to form three limbs—an ascending, transverse and descending—which latter limb passes posteriorly to the rectum. In Fig. 2 the empty ali-

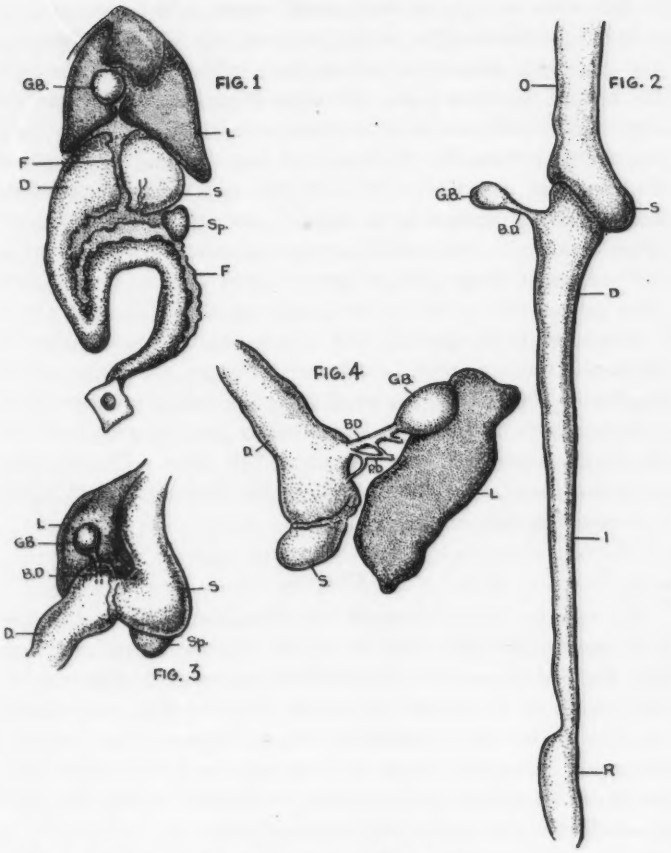


FIG. 1. Diagram of the alimentary tract *in situ*.

FIG. 2. Scheme of the alimentary tract, dissected free from surrounding structures.

FIG. 3. Shows the entrance of the bile duct into the duodenum.

FIG. 4. Diagram to show the pancreatic duct entering the duodenum in close relation to the bile duct. *L.* Liver; *G.B.* Gall bladder; *B.D.* Bile duct; *P.D.* Pancreatic duct; *Sp.* Spleen; *F.* Fat; *O.* Œsophagus; *S.* Stomach; *D.* Duodenum; *I.* Intestine; *R.* Rectum.

mentary canal dissected free from surrounding structures is stretched out to show the length of the various portions and their relation to each other.

The liver is a large three-lobed organ which occupies an anterior ventral position in the abdomen, the left lobe obscuring the œsophagus and part of the stomach, while the tip of the right lies behind the duodenum. Between these lobes is situated the large gall bladder, which in the specimen examined had a body length of 14 mms. by 10 mms. and was drained by a duct of 8 mms. (Figs. 4 and 1). This duct pierced the duodenum on its anterior dorsal surface a few mms. below the pyloric sphincter (Figs. 2 and 3). No special microscopic examination was made for pancreatic tissue, but it appears that the pancreatic duct joins the bile duct or enters the duodenum close to it (Fig. 4).

According to Krüger (1), in a closely related species *Zoarces viviparus*, the pancreas is diffused amongst the fatty tissue situated in the loops of the small intestine and forms pancreatic threads which lie in the groove between the stomach and the duodenum, and also cover the short bile duct. The opening from the pancreatic gland, in this species, is combined with the bile duct, but does not empty into it.

## Methods.

The reaction of the stomach, duodenum and bile in the eel-pout and other fishes was measured by the "spot" method described by Felton (2). This method was very satisfactory for the purpose, as it required only a few drops of fluid for a determination. The values obtained from it, however, can only be considered as approximate, as a protein and salt error was frequently introduced by the presence of mucus or partially digested food in the sample under investigation.

To study the digestive enzymes in the eel-pout, extracts were made of different parts of the alimentary tract. The method of making these extracts was as follows: The mucous membrane was washed in running water, scraped off from the muscular layers and made into a brew consisting of 50 per cent. tissue, 50 per cent. extracting substance and toluene. The mixture was allowed to stand for two days at room temperature, after which it was filtered before adding to the substrate. Saline, 30 per cent. alcohol, and glycerol were all used for extracting substances, but were not all equally effective, the alcoholic extract yielding

the most active amylase and lipase, while glycerol only extracted a protease. The latter enzyme was rather weak and difficult to obtain under any of the observed conditions.

In the experiments to test the presence of an amylase, 2–4 drops of the filtered enzyme extract were added to 2 cc. 5 per cent. soluble starch, the $P_H$ adjusted between 6.6 and 7.0 and the whole incubated at 37° C. During the incubation, the mixture was tested occasionally with iodine to ascertain how the digestion was proceeding, and at the end of two days a quantitative sugar determination was carried out by the Folin-Wu method (3) for blood sugar.

For lipase determinations the method of Anrep Lush and Palmer (4) was used. Three drops of the enzyme extract were added to a solution of glycerol triacetate buffered at a $P_H$ 8.0. The time was taken which was required for the enzyme to turn the solution to a $P_H$ 7.0 at room temperature. Changes in reaction were observed by noting the color changes in an indicator which had been added, at the beginning, to the mixture.

Both the amylase and lipase experiments were controlled by comparing with similar experiments in which the extracts had been previously boiled for 10 minutes.

A quantitative method was not used for the estimation of a protease. The presence of this enzvme was determined by the digestive action of the extract on protein. For this purpose fibrin was obtained from fresh blood and kept in glycerol, being thoroughly washed, and dried between filter paper, before using. Small shreds were then placed in three tubes, each containing some of the extract to be tested, and their reaction adjusted to a $P_H$ 2.0, $P_H$ 6.8 and $P_H$ 8.0 respectively. These mixtures were incubated at 37° C. and the time noted which was taken to digest the fibrin.

### *Experimental data*

*The Reaction in the Alimentary Tract of the Eel-Pout under Different Conditions.*—Investigations to determine the reaction of the stomach and duodenum were carried out on a large number of eel-pouts. These determinations were made, either immediately after the fish had been hooked on the trawl or after they had been kept in a tank or cage for several days without food.

Thus, it was possible to study the reaction in the alimentary canal under different conditions in both fasting and fed animals. In the Bay of Fundy the eel-pouts live chiefly on mytilus, periwinkles, scallops, barnacles, sea urchins and occasionally fish; they will, however, bite herring as readily as clam bait.

To obtain a sample for a $P_H$ determination, the abdomen was first opened by a mid-line incision, the alimentary tract removed intact, and a ligature placed between the stomach and duodenum to prevent the passage of fluid from one to the other during the manipulations. A few drops of the contents were then sucked out by a pipette and were diluted 2–4 times before measuring the $P_H$. If the fish under examination was in a starving condition and the alimentary tract empty, the mucous membrane was washed with a few drops of distilled water on which the $P_H$ was subsequently determined.

The following table shows the reaction of the stomach, duodenum and bile under different conditions.

TABLE I.

REACTION OF THE STOMACH, DUODENUM AND BILE IN THE EEL-POUT.

*A. Stomach Empty.*

| Fish Number. | Stomach. | | Duodenum. | | Bile. | |
|---|---|---|---|---|---|---|
| | Contents. | $P_H$. | Contents. | $P_H$. | Color. | $P_H$. |
| 10 | Empty | 7.0 | Empty | 8.0 | Greenish yellow | 5.8 |
| 13 | Empty | 5.8 | Yellow mucus | 6.8–8.4 small intest. | Greenish yellow | 5.6 |
| 14 | Empty | 7.0 | Empty | 6.4 | Greenish yellow | 5.4 |
| 19 | Empty | 8.6 | Fluid and mucus | 7.0 | Greenish yellow | 5.8 |
| 27 | Empty | 6.5 | Empty | 8.4 | Light greenish yellow | 5.4 |

*B. Stomach, Fluid Contents Only.*

| | | | | | | |
|---|---|---|---|---|---|---|
| 9 | Yellow fluid | 7.4 | Clear fluid | 7.7 | Greenish yellow | 5.8 |
| 25 | Yellow fluid | 8.2 | Yellow fluid | 8.2 | Dark greenish yellow | 5.8 |
| 26 | Colorless fluid | 8.4 | Fluid, hermit crab | 8.0 | Dark greenish yellow | 5.6 |

*C. Stomach Full.*

| | | | | | | |
|---|---|---|---|---|---|---|
| 2 | Clam bait | 8.0 | Small shell fish | 8.3 | Dark green | 5.4 |
| 15 | Clam bait | 6.6 | Brown fluid, shell fish, lower in intest. | 7.4<br>8.2<br>lower in intest. | — | — |
| 1 | Fish | 7.1 | — | 6.8 | — | — |
| 5 | Herring bait | 6.4 | Empty | 7.4 | Dark green | 5.4 |
| 8 | Herring, hermit crab | 6.6 | Snails, hermit crab | 7.2<br>7.6<br>lower in intest. | — | — |
| 3 | Fish and snails | 7.6 | Empty | 7.0 | Light greenish yellow | 4.7 |
| 4 | Small clams, scallops and ascidians partially digested | 8.4 | Small shell fish partially digested | 8.2 | Light greenish yellow | 6.3 |

*Reaction in the Alimentary Tract of Living Eel-Pout with and without Forced Feeding.*—To investigate further the reaction of the stomach a series of experiments was performed on living eel-pouts during periods of fasting and after forced feeding. In order to obtain a sample from the stomach without killing the fish, a long glass tube was inserted through the œsophagus and some of the contents sucked out. As this process involved considerable manipulation, causing a certain amount of injury to the animal, these experiments did not give the expected results. They confirmed, however, the findings on recently killed fish.

As it was thought possible that the reaction in the stomach might, to a certain extent, be determined by the food ingested, various substances such as clam, flounder and sea urchin were given in the forced feedings. The reactions of these foods differ somewhat, flounder flesh having a $P_H$ of 6.8, clam $P_H$ about 6.8 and ground-up sea urchin (including the shell) a $P_H$ of 8.2.

The experiments of Tables I. and II. show that the reaction in the stomach of starved fishes as well as in fed animals is, as a rule, near the neutral point. It may, however, range slightly on either side of this point, the alkaline reaction even reaching a $P_H$ of 8.5.

Two tentative explanations may be offered for this phenomenon. It is possible that the alkalinity of the gastric contents may be

due to the alkalinity of the food (shell fish), or it may be the result of the regurgitation of alkaline duodenal juices into the stomach. There is evidence for the latter suggestion, because in two instances the stomach contents showed a positive test for bile, this indicating the presence of duodenal juices.

The most striking fact, however, in all these experiments is that none of the food stuffs or even such a strong gastric stimulant as alcohol produced a high acidity in the stomach.

## TABLE II.

$P_H$ OF SAMPLES TAKEN FROM THE STOMACH OF LIVING EEL-POUTS.

*A. Without Feeding.*

| No. Fish. | Date. | Interval between Samples. | $P_H$. | Description of Sample. |
|---|---|---|---|---|
| 7 | July 29 | 0 hrs. | 7.0 | Clear watery. |
| | | 3 hrs. | 7.0 | Clear watery. |
| | July 30 | 18 hrs. | 8.2 | Mucus, slightly opaque. |
| | | 4 hrs. | 7.4 | Mucus, slightly opaque. |
| | | 18 hrs. | 7.2 | |
| 17 | Aug. 16 | 0 hrs. | 8.5 | Reddish brown fluid, sea urchin shell. |
| 18 | Aug. 16 | 0 hrs. | 8.3 | Reddish brown fluid. |

*B. Sea Urchin Fed.*

| | | | | |
|---|---|---|---|---|
| 23 | Aug. 19 | 0 hrs. | 7.2 | Clear mucus. |
| | | 2 hrs. | Sea urchin fed $P_H$ 8.2. | |
| | | 7 hrs. | 8.0 | Reddish fluid cont. sea urchin. |
| | Aug. 20 | 16 hrs. | 7.0 | Mucus. |

*C. Clam Fed.*

| | | | | |
|---|---|---|---|---|
| 6 | July 27 | 0 hrs. | 8.4 | — |
| | | 17 hrs. | 7.4 | — |
| | | 7 hrs. | 8.4 | — |
| | | 17 hrs. | 6.8 | Mucus. |
| | | Clam fed $P_H$ 6.8. | | |
| | | 4 hrs. later | 5.8 | Mucus. |
| | | 3 hrs. | 7.6 | — |
| 12 | Aug. 3 | 0 hrs. | 7.0 | Clear watery. |
| | | 24 hrs. | 4.4 | Gray mucus. |
| | | Clam fed $P_H$ 6.6. | | |
| | | 3 hrs. | 6.6 | — |
| | | 16 hrs. | 4.7 | — |
| | | 5 hrs. | 6.6 | Died. |

TABLE II. (*Continued*).

*D. Flounder and Alcohol Fed.*

| No. Fish. | Date. | Interval between Samples. | $P_H$. | Description of Sample. |
|---|---|---|---|---|
| 20 | Aug. 20 | 0 hrs. | 8.6 | Clear fluid. |
| | | 1 hr. | Flounder fed $P_H$ 6.8. | |
| | | 8 hrs. | 8.0 | Clear, contained pieces flounder. |
| | Aug. 21 | 16 hrs. | 7.6 | Clear. |
| | Aug. 22 | 48 hrs. | 6.2 | Clear. |
| | Aug. 24 | 48 hrs. | 6.6 | Clear. |
| | | 48 hrs. | 10 cc. 10 per cent. alcohol fed $P_H$ 6.6. | |
| | | 3 hrs. | 6.6 | — |
| 21 | Aug. 19 | 0 hrs. | 6.4 | |
| | | 1 hr. | Flounder fed $P_H$ 6.8. | |
| | | 8 hrs. | 6.6 | — |
| | Aug. 20 | 16 hrs. | 4.4 | — |
| | Aug. 22 | 48 hrs. | 6.4 | — |
| | Aug. 24 | 48 hrs. | 6.5 | — |
| | | 48 hrs. | 10 cc. 10 per cent. alcohol $P_H$ 6.6. | |
| | | 1 hr. | 7.4 | — |
| | | 1 hr. | 7.4 | — |
| | | 1 hr. | 10 cc. 10 per cent. alcohol $P_H$ 6.6. | |
| | | 1 hr. | 7.2 | — |
| | | 3 hrs. | 6.4 | — |

*E. Alcohol Fed.*

| | | | | |
|---|---|---|---|---|
| 24 | Aug. 19 | 0 hrs. | 6.2 | |
| | | 2 hrs. | Fed sea urchin $P_H$ 7.6. | |
| | | 7 hrs. | 7.6 | — |
| | Aug. 20 | 16 hrs. | 6.4 | Mucus clear. |
| | Aug. 22 | 24 hrs. | 5.4 | Mucus clear. |
| | Aug. 24 | 24 hrs. | 7.8 | Mucus clear. |
| | | 24 hrs. | Gave alcohol 10 cc. 10 per cent. $P_H$ 6.8. | |
| | | 1 hr. 30 min. | 8.0 | |
| | | | Gave alcohol 10 cc. 10 per cent. $P_H$ 6.8. | |
| | | 35 mins. | 6.8 | — |
| | | 3 hrs. | 8.6 | — |
| 27 | Aug. | 0 hrs. | 8.2 | Yellowish fluid. |
| | | | Gave 10 cc. 10 per cent. alcohol $P_H$ 6.8. | |
| | | 20 mins. | 7.2 | |
| | | 4 hrs. | 8.0 | |

*Reaction in the Alimentary Tract of Other Fishes.*—The reaction of the stomach and duodenum of various fishes was investigated so that they could be compared with the eel-pouts. All the examined fishes, except fundulus, had a neutral or slightly acid reaction in the stomach during fasting, and a very high acidity during digestion, as is shown in the following table.

## TABLE III.

REACTION OF THE STOMACH, DUODENUM AND BILE IN VARIOUS FISHES.

*A. Stomach Empty.*

| Fish Specimen. | Stomach. | | Duodenum. | | Bile. |
|---|---|---|---|---|---|
| | Contents. | $P_H$. | Contents. | $P_H$. | $P_H$. |
| Skate (Barn-door)..... | Empty | 1.5 Cardiac<br>3.7 Pyloric | Empty | 6.3 Duoden.<br>7.2 Intest. | — |
| (*a*) Fundulus | No stomach | — | Empty | 7.1 | 7.0 |
| (*b*) | No stomach | — | Empty | 7.2 | 6.8 |
| (*c*) | No stomach | — | Empty | 7.4 | 7.0 |
| (*a*) Flounder. | Empty | 6.8 | Empty | 8.4 | — |
| (*b*) | Empty | 6.4 | Empty | 8.0 | 6.6 |
| Haddock..... | Empty | 6.2 | Full small shell fish | 6.7 | 6.4 |
| Herring..... | Empty | 6.8 | Empty | 7.0 | 5.8 |
| Lump fish.... | Empty | 4.0 | Empty | 8.6 | — |
| Sculpin..... | Empty | 7.4 | Empty | — | 5.8 |

*B. Stomach Full.*

| | | | | | |
|---|---|---|---|---|---|
| Skate..... | Full herring bait (beginning of digestion) | 5.2 Cardiac<br>5.5 Pyloric | Full herring bait | 6.3 Duoden.<br>7.2 Intest. | 5.6 |
| Skate..... | Full herring (partially digested) | 2.8 | Fluid | 4.6 | 5.8 |
| Lump fish.... | Digesting food | 2.8 | Digesting food | 8.2 | — |
| Sculpin..... | Full fish (digesting) | 2.2 | Fluid | 8.4 | 5.4 |
| Sculpin..... | Full herring bait (digesting) | 2.8 | Digesting food | 7.4 Duoden.<br>8.2 Intest. | 5.6 |

It will be seen, on comparison of the above table with Tables I. and II., that the gastric digestion of the eel-pout presents certain pecularities, being different from the usual type of acid gastric digestion in fishes. Since there are fishes deprived of a stomach (*e.g.*, the family of Cyprinidæ, *Fundulus*, etc.) the question arises as to whether the eel-pout possesses an organ which is capable of secreting pepsin-hydrochloric acid. From an anatomical point of view, there is no doubt that the eel-pout possesses a structure corresponding to a stomach. Although this diverticulum is rather small, it cannot be considered as part of duodenum, for it is separated from it by a sphincter. Further evidence for this lies in the fact that the common bile and pancreatic duct enters the duodenum just below this sphincter.

As no histological examination could be made of the microscopic structure of the glands in the mucous membrane of the stomach, further investigation was restricted to a study of the digestive enzymes in the gastric and duodenal contents, and in extracts made from different parts of the alimentary canal.

*Digestive Enzymes in Extracts of the Stomach, Duodenum and Liver of the Eel-Pout.*—In Table IV., three typical experiments are quoted to show the amylolytic, lipolytic and proteolytic action of differently prepared extracts of the gastric and duodenal mucous membrane and of the liver. A number of other experiments gave analogous results.

As may be seen from Table IV., the presence of a lipase was demonstrated in the extracts of the liver and in the mucous membrane of the stomach and duodenum of the eel-pout. The activity of this enzyme was most pronounced in the alcoholic liver extracts, which were frequently strong enough to produce a $P_H$ change from 8.0 to 7.0 in 1 hour and 30 minutes; compared with this, its action in the stomach is somewhat weaker, 4 hours being the average time taken to produce the desired change in reaction. The lipolytic enzyme of the duodenum is the weakest of the three, since it is usually one or more hours slower in its action than that of the stomach.

The strongest amylase was found in the extracts of the mucous membrane of the duodenum, which gave values as high as .6 per cent. with the Folin-Wu method. On the other hand extracts of the stomach had a very weak, if any, amylolytic action, .12 per cent. being the highest figure obtained. As the liver extract itself was found to contain glucose, the high values procured in the sugar determination are useless. There is, however, some slight evidence for the presence of an amylase in the liver because in several of the experiments there is an indication of a color change in the starch following the addition of iodine.

The range of the Folin-Wu method is from .07–.4 per cent. In the cases where the values obtained are higher than .4 the solution had been previously diluted so as to come in the correct range. The value obtained was then multiplied by the dilution. Controls done on soluble starch without extracts gave an average value of .076 per cent.

## TABLE IV.

ENZYMES IN EXTRACTS OF THE MUCOUS MEMBRANE OF THE STOMACH, DUODENUM AND LIVER.

| Date. | No. Fish. | Part Dig. Tract Used for Extracts. | Amylase. | Lipase. | Protease. |
|---|---|---|---|---|---|
| Aug. 5 | 13 | Stomach, alcohol ext. | 2 cc. starch + 4 drops ext.<br>1 dy. iodine reac.—blue.<br>2 dys. iodine reac.—blue.<br>2 dys. sugar deter. .091% −.<br>Boiled control = .069% −. | 3 drops ext. + buffer at $P_H$ 8.0.<br>Went to $P_H$ 7.0 +.<br>Control $P_H$ 8.0 −. | 1. fib. + 15 drops ext. at $P_H$ 1.6 −.<br>2. fib., 15 drops ext. at $P_H$ 6.0 −.<br>3. fib., 15 drops ext. at $P_H$ 8.0 −.<br>3 days no digestion. |
| | | Duodenum, alcohol ext. | 2 cc. starch + 4 drops ext.<br>1 dy. iodine reac.—blue.<br>2 dys. iodine reac.—light blue.<br>2 dys. sugar deter. = .60% +. | 3 drops ext. + buffer at $P_H$ 8.0.<br>7 hrs. went to $P_H$ 7.0 +.<br>7 hrs. control $P_H$ 8.0 −. | 1. same as above, $P_H$ 1.6 +.<br>2. same as above 15, $P_H$ 6.0 −.<br>3. same as above 15, $P_H$ 8.0 −.<br>3 dys. Nos. 2 and 3, no diges.<br>3 dys. No. 1 digested. |
| | | Liver, alcohol ext. | 2 cc. starch + 2 drops ext.<br>2 dys. iodine reac.—blue.<br>Sugar deter. useless, as liver ext. cont. sugar. | 3 drops ext. + buffer at $P_H$ 8.0.<br>7 hrs. went to $P_H$ 7.0 +.<br>7 hrs. control $P_H$ 8.0 −. | 1. same as above, $P_H$ 1.6 −.<br>2. same as above, $P_H$ 6.0 −.<br>3. same as above, $P_H$ 8.0 −.<br>3 dys., no digestion. |
| 8 | 14 | Stomach, glycerol ext. | 2 cc. starch + 2 drops ext.<br>1 dy. iodine reac.—blue.<br>2 dys. iodine reac.—blue.<br>2 dys. sugar deter. = .08% −.<br>Boiled control = .03% −. | 3 drops ext. + buffer at $P_H$ 7.0.<br>3 dys. went to $P_H$ 7.0.<br>(Glycerol ext. not good for lipase). | 1. fib., 5 drops ext.<br>20 drops $H_2O$, $P_H$ 1.6 +.<br>2. fib. and same, $P_H$ 6.0 −.<br>3. fib. and same, $P_H$ 8.0 −.<br>1 dy. Nos. 2 and 3, no diges.<br>1 dy. No. 1, digested. |
| | | Duodenum, glycerol ext. | 2 cc. starch + 2 drops ext.<br>1 dy. iodine reac.—blue.<br>2 dys. iodine reac.—light blue.<br>2 dys. sugar deter. = .20% −.<br>Boiled control = .04% −. | 3 drops ext. + buffer at $P_H$ 8.0.<br>3 dys. went to $P_H$ 7.0. | 1. same as above, $P_H$ 1.6 −.<br>2. same as above, $P_H$ 6.0 −.<br>3. same as above, $P_H$ 8.0 −.<br>3 dys., no digestion. |

TABLE IV. (*Continued*).

| Date. | No. Fish. | Part Dig. Tract Used for Extracts. | Amylase. | Lipase. | Protease. |
|---|---|---|---|---|---|
| 18 | 19 | Stomach, HCl ext. | No amylase in HCl ext. | No lipase in HCl ext. | 1. fib. + 10 drops ext., 10 drops $H_2O$, $P_H$ 2.0 −.<br>2. fib. same boiled. 10 drops $H_2O$, $P_H$ 2.0 −.<br>3 dys., no digestion. |
| | | Duodenum, alcohol ext. | 2 cc. starch + 4 drops ext.<br>1 dy. iodine reac.—blue.<br>2 dys. iodine reac.—very pale.<br>2 dys. sugar deter. = .23% +.<br>2 dys. boiled control = .08% −. | 3 drops ext. + buffer at $P_H$ 8.0.<br>3 hrs. went to $P_H$ 7.0 +.<br>3 hrs. control $P_H$ 8.0 −. | 1. fib. + 15 drops ext., 10 drops $H_2O$, 2.0 −.<br>2. fib. same, $P_H$ 6.0 −.<br>3. fib. same, $P_H$ 8.0 −.<br>3 dys., no digestion. |
| | | Liver, alcohol ext. | 2 cc. starch + 4 drops ext.<br>2 dys. iodine reac.—blue.<br>Sugar deter. useless. | 3 drops ext. + buffer at $P_H$ 8.0.<br>1½ hrs. went to $P_H$ 7.0 +.<br>1½ hrs. control $P_H$ 8.0 −. | 1. fib. + 15 drops ext., 15 drops $H_2O$, $P_H$ 2 −.<br>2. fib. same, $P_H$ 6.0 −.<br>3. fib. same, $P_H$ 8.0 −.<br>3 dys., no digestion. |

\+ at end of line means digestion.

− at end of line means no digestion.

There is no proteolytic enzyme in the extracts of the liver or of the duodenum, but occasionally such a one was found in the extracts of the stomach. This enzyme could be extracted with glycerol only, and acted slowly, in a very acid medium $P_H$ 1.6–2.0. Since there is no difficulty whatever in obtaining pepsin from the mucous membrane of fishes possessing an acid gastric digestion, by means of such extracting substances as glycerol, .36 per cent. to .4 per cent. HCl or even distilled water (Kenyon) (5), the results quoted in this investigation are worthy of attention. The conclusion must be drawn that the acid-peptic digestion does not play an important part in the alimentary canal of the eel-pout. Even if an acid gastric juice is secreted its pepsin has very little chance to act either in the stomach or the duodenum. This is clear from the fact that the usual range of $P_H$ in the stomach of the eel-pout is from 6.4 to 8.2; in a few instances only, it was found to be as low as 4.0 to 4.4 (Table II). The reaction in the duodenum is always on the alkaline side.

Further experiments dealing with the estimation of digestive enzymes in the juices taken from the stomach and intestine of various eel-pouts lend a strong support to the above view.

One may see from Table V. that in the juice taken from the stomach of a number of animals a very strong proteolytic enzyme was found which digested fibrin at a $P_H$ 6.4 to 8.2. This enzyme could not be extracted either from the gastric or duodenal mucous membranes. A possible explanation of this phenomenon is the assumption that the pancreatic juice regurgitates from the duodenum and continues its action in the stomach.

## Discussion.

The gastric digestion in the eel-pout presents marked peculiarities. Although a protein-digesting enzyme could be extracted from the gastric mucous membrane, it appears that it cannot play a great part in gastric digestion. The evidence for this is as follows: This proteolytic enzyme is not easily extracted by the usual methods, which is an indication that the mucous membrane is not rich in its contents. It seems, however, to be a true digestive enzyme, because it is active at a $P_H$ 1.6 to

## TABLE V.

DIGESTIVE ENZYMES IN THE JUICE TAKEN FROM THE STOMACH AND INTESTINE OF THE EEL-POUT.

| Fish No. | Juice Taken from | Amylase. | Lipase. | Protease. |
|---|---|---|---|---|
| 15 | Stomach | 2 cc. 5% starch, 5 drops juice.<br>1 dy. iod. test—pale lavender.<br>1 dy. sugar deter. .66% ++. | 3 drops ext. buffer at $P_H$ 8.0. Over night passed $P_H$ 7.0 ++. | Juice and fibrin.<br>5 hrs. all digested, $P_H$ 6.4 ++. |
| | Duodenum | 2 cc. 5% starch, 5 drops juice.<br>1 dy. iod. test—colorless.<br>1 dy. sugar deter. .83% ++. | 3 drops ext. buffer at $P_H$ 8.0. Over night went to $P_H$ 7.0 +. | Juice and fibrin.<br>5 hrs. all digested, $P_H$ 6.4 ++. |
| 17 | Stomach | —— | —— | Juice and fibrin ($P_H$ 8.5).<br>4¼ hrs. all digested ($P_H$ 7.2) ++. |
| 18 | Stomach | —— | —— | Juice and fibrin ($P_H$ juice 8.3).<br>4 hrs. all digested ($P_H$ 6.6) ++. |
| 25 | Duodenum | —— | —— | Juice and fibrin ($P_H$ juice 8.2).<br>2 hrs. all digested ($P_H$ at 8.2) +++. |
| 26 | Stomach | 4 cc. 5% starch, 12 drops gastric juice.<br>1 dy. sugar deter. .15% +. | 3 drops ext. buffer at $P_H$ 8.0.<br>18 hrs. went to $P_H$ 7.0 +. | Juice and fibrin ($P_H$ juice 8.4).<br>1¾ hr. all digested ($P_H$ at 7.4) +++. |
| | Intestine (below duod.) | 4 cc. 5% starch, 12 drops juice.<br>1 dy. sugar deter. .40% +. | 3 drops ext. buffer at $P_H$ 8.0<br>24 hrs. still at $P_H$ 8.0 −. | Juice and fibrin ($P_H$ juice 8.0).<br>12 hrs. all digested +. |

*Remark.*—Glycerol extracts were made of the stomach and duodenum of fish No. 15; no proteolytic enzyme was present in the duodenal extract and in the extract of the stomach fibrin was digested only at a $P_H$ 2.0, and not at a $P_H$ 6.0 or 8.0, though the juice from the stomach readily digested fibrin at $P_H$ 6.4.

2.0, while the autolytic enzyme of a type of pepsin is destroyed at a $P_H$ of 2.6 (Bradley) (6). Moreover, the action of this enzyme on such an easily digestible substrate as fibrin is rather weak.

Further evidence that the proteolytic enzyme is not important in gastric digestion lies in the fact that only occasionally was the reaction of the stomach found to be acid enough ($P_H$ 4.0–5.8) to allow a pepsin-like enzyme to act. According to McFarlane (7) the range of activity for pepsin is from a $P_H$ of 0.5 to 6.5.

These facts show that if peptic digestion takes place in the stomach of the eel-pout it plays a subordinate or secondary part. The protein substances in the food of this animal are digested chiefly by the trypsin of the pancreatic juice, which probably regurgitates into the stomach.

It would be highly interesting to perform a histological examination of the gastric mucous membrane of the eel-pout to determine whether the peptic glands are fully developed as to both number and structure.

## SUMMARY.

1. The eel-pout has a true anatomical stomach. The common bile and pancreatic duct opens into the duodenum below the pyloric sphincter.

2. The reaction of the stomach in both fasting and fed animals is near the neutral point, ranging from a $P_H$ of 6.5 to 8.4. The reaction in the duodenum is slightly alkaline.

3. The extracts of the stomach possess a strong lipase, a very weak amylase and a pepsin-like enzyme, digesting at a $P_H$ of 1.2. The duodenal extracts, on the other hand, have a strong amylase, a lipase and no protease. Liver extracts had a marked lipolytic action, but contained no protease.

4. Digestive juices removed from both the stomach and duodenum of eel-pouts had a lipase, amylase and a very strong proteolytic enzyme, digesting at a $P_H$ near the neutral point.

My thanks are due to Dr. B. Babkin, who directed this work, to the Biological Board of Canada for the permission to use the facilities of the Atlantic Biological Station, St. Andrews, N. B., and to Dr. A. G. Huntsman and his staff for valuable assistance received in the course of the study.

## BIBLIOGRAPHY.

1. **Krüger, A.**
'05 Wissenschaftliche Meeruntersuchungen Abt. Kiel, N.F., Vol. 8, p. 57.
2. **Felton, L. D.**
'21 Jour. Biol. Chem., Vol. 46, p. 229.
3. **Folin, I., and Wu, H.**
'20 Jour. Biol. Chem., Vol. 41, p. 367.
4. **Anrep, Lush and Palmer.**
'25 Jour. Physiol., Vol. 59, p. 436.
5. **Kenyon, W. R.**
'25 U. S. Bull. Bureau of Fisheries, Vol. 41, p. 181.
6. **Bradley, H. C.**
'22 Jour. Biol. Chem., Vol. 52, p. 467.
7. **McFarlane, J., et al.**
'27 Jour. Gen. Physiol., Vol. 10, p. 437.

# NOTE ON THE BILE IN DIFFERENT FISHES.

MARGARET E. MacKAY.

*(From the Atlantic Biological Station, St. Andrews, N. B.)*

The results of investigations on two problems concerning the bile of different fishes of the northeast Atlantic coast will be presented in this note: (1) A series of determinations were carried out on the reaction of gall-bladder bile in various fishes; (2) The content of enzymes in the gall-bladder bile of *Fundulus Heteroclitus* was studied.

## The Reaction of Gall-Bladder Bile.

There is a definite difference between the reaction of the gall-bladder and hepatic bile in warm-blooded animals, the first being less alkaline than the second. In most of the animals observed (ox, pig, sheep, rabbit and guinea-pig), Neilson and Meyer (1), and Tschopp (2), found the reaction of gall-bladder bile to be slightly on the alkaline side of neutrality, the average $P_H$ range being 7.0–7.5. Okada (3) has shown that in the dog the reaction of the gall-bladder bile is on the acid side ($P_H$ 6.39–6.74), while that of hepatic bile is decidedly more alkaline ($P_H$ 7.89–8.10). Brugsch and Horster (4) found a $P_H$ of 7.4–7.89 for bile received from a dog with a permanent fistula of the gall bladder. In this animal the bile could not remain long in the gall bladder and it is interesting to note that its reaction is only a little less alkaline than that found by Okada for hepatic bile.

In contrast to the neutral or slightly alkaline bile of warm-blooded animals, it was found that in the fishes investigated the bile though sometimes neutral was usually on the acid side. The degree of acidity varied considerably in different species but was fairly constant in a given species.

The colorimetric "spot" method elaborated by Felton was used for determining $P_H$. For this method it was first necessary to dilute the bile from three to eight times, depending on the depth of its color. As bile is a buffered solution this dilution would not cause any appreciable error in the determination.

The following table shows the $P_H$ of the bile in all the fishes examined. In some cases where the bile was very dark in color, it was difficult to make a comparison even after diluting eight times.

TABLE I.

THE $P_H$ OF THE GALL-BLADDER BILE IN DIFFERENT FISHES.

| Fish. | Color of Bile. | Reaction. |
|---|---|---|
| Fundulus (*Fundulus Heteroclitus*)..... | Emerald green | 7.0 |
| Fundulus (*Fundulus Heteroclitus*)..... | Emerald green | 6.8 |
| Fundulus (*Fundulus Heteroclitus*)..... | Emerald green | 7.0 |
| Fundulus (*Fundulus Heteroclitus*)..... | Emerald green | 6.9 |
| Eel-Pout (*Zoarces Anguillaris*)..... | Greenish yellow | 5.4 |
| Eel-Pout (*Zoarces Anguillaris*)..... | Greenish yellow | 5.4 |
| Eel-Pout (*Zoarces Anguillaris*)..... | Greenish yellow | 5.8 |
| Eel-Pout (*Zoarces Anguillaris*)..... | Greenish yellow | 6.2 |
| Eel-Pout (*Zoarces Anguillaris*)..... | Dark greenish yellow | 5.4 |
| Eel-Pout (*Zoarces Anguillaris*)..... | Dark greenish yellow | 5.4 |
| Eel-Pout (*Zoarces Anguillaris*)..... | Light greenish yellow | 5.6 |
| Eel-Pout (*Zoarces Anguillaris*)..... | Dark greenish yellow | 5.4 |
| Eel-Pout (*Zoarces Anguillaris*)..... | Light greenish yellow | 5.4 |
| Eel-Pout (*Zoarces Anguillaris*)..... | Light greenish yellow | 5.8 |
| Eel-Pout (*Zoarces Anguillaris*)..... | Light greenish yellow | 5.8 |
| Eel-Pout (*Zoarces Anguillaris*)..... | Light greenish yellow | 5.6 |
| Eel-Pout (*Zoarces Anguillaris*)..... | Light greenish yellow | 5.4 |
| Skate (*Raja erinacea*)..... | Greenish yellow | 7.6 |
| Skate (*Raja erinacea*)..... | Dark green | 6.4 |
| Skate (*Raja erinacea*)..... | Greenish yellow | 6.7 |
| Skate (*Raja erinacea*)..... | Light greenish yellow | 5.4 |
| Skate (*Raja erinacea*)..... | Dark green | 5.8 |
| Skate (*Raja erinacea*)..... | Dark green | 5.6 |
| Skate (*Raja erinacea*)..... | Greenish yellow | 6.8 |
| Sculpin (*Cottus grænlandicus*)..... | Colorless | 5.8 |
| Sculpin (*Cottus grænlandicus*)..... | Pale green | 5.4 |
| Sculpin (*Cottus grænlandicus*)..... | Pale green | 5.8 |
| Haddock (*Melanogrammus*)..... | Dark green | 6.4 |
| Herring (*Clupea Harengus*)..... | Emerald green | 5.8 |
| Flounder (*Paralichtys dentatus*)..... | Dark green | 6.6 |
| Flounder (*Paralichtys dentatus*)..... | Dark green | 7.0 |

## A STUDY OF THE SOURCE OF THE ENZYMES IN THE BILE OF *FUNDULUS HETEROCLITUS*.

Babkin and Bowie (5) found proteolytic, amylolytic and lipolytic enzymes in the bile of *Fundulus*. This bile was collected by squeezing the gall bladder and it was possible that the enzymes contained in it came from a layer of pancreatic tissue which they later found surrounding this viscus. Therefore to investigate the source of these enzymes it was necessary to use a method for emptying the gall bladder which would prevent the bile coming in contact with the outer pancreatic cells.

The first method used was as follows: The bile duct was

ligated and the gall bladder removed and dipped in warm melted wax. When this had hardened slightly, a hot needle was used to puncture the viscus and the bile forced out by slight pressure.

This method was unsatisfactory as the bile still contained both amylolytic and proteolytic enzymes.

Another method was therefore devised for emptying the gall bladder. The viscus was first removed, as previously described, and placed for one minute in a solution of Bouin's fluid containing picric acid, acetic acid and formalin. The purpose of this treatment was to kill the surrounding layer of pancreatic cells. The bladder was next washed a number of times in distilled water; it was then punctured with a hot needle and its contents squeezed out.

It was found that bile collected in this way had no enzymes. The results of two experiments are quoted below.

TABLE II.

| Date. | No. of Fish Used. | Amylase. | Lipase. | Protease. |
|---|---|---|---|---|
| Aug. 13... | Bile from 6 fish. | 2 cc. 5% starch. 2 drops bile. 2 days iod. test, blue—no sugar. | 3 drops bile buffer at $P_H$ 8.0. 2 days—no change in $P_H$. | |
| Aug. 23... | Bile from 10 fish. | 2 cc. 5% starch. 2 drops bile. 2 days—no sugar. | 3 drops bile buffer at $P_H$ 8.0. 2 days—no change in $P_H$. | 10 drops bile, duod. extract, fibrin. 2 days—no digestion. |

The method for lipase is that of Anrep, Lush and Palmer.

An objection could be made to these results on the grounds that Bouin's fluid may have penetrated the wall of the gall bladder and killed any enzymes which were present in the bile. This, however, is rather unlikely; it is more probable that in Babkin and Bowie's experiments the pancreatic enzymes were mixed with the sterile bile during its collection from the gall bladder. Therefore, their conclusion is valid that the pancreatic gland of fundulus produces trypsin, amylase and lipase.

My thanks are due to Dr. Babkin who directed this work and to Dr. Huntsman and the Biological Board of Canada for use of the facilities of the Atlantic Biological Station.

BIBLIOGRAPHY.

1. **Neilson and Meyer.**
'21 Journ. of Infect. Dis., Vol. 28, p. 510.
2. **Tschopp.**
'25 Zentralblatt f. Allg. Pathol. u. pathol. Anat., Vol. 36, S. 123.
3. **Okada.**
'15–'16 Journ. of Physiol., Vol. 50, p. 114.
4. **Brugsch and Horster.**
'24 Med. Klinik, No. 20.
5. **Babkin and Bowie.**
'28 BIOL. BULL., Vol. 54, p. 254.

# THE EFFECT OF NEUROPHIL DRUGS ON THE FIDDLER CRAB, *UCA PUGNAX*.

OSCAR W. RICHARDS.

*(From the Department of Biology, Clark University, Worcester, and Marine Biological Laboratory, Woods Hole.)*

These experiments were made to determine if the fiddler crab, *Uca pugnax* S. I. Smith, could be forced to move in only one direction, rather than sidewise, forward and backward, by means of the effect of a suitable drug. If this could be accomplished it would simplify the study of the phototropism of this animal. A drug that causes the legs on the opposite sides to pull against each other, such as nicotine, phenol, or veratrine, leads inevitably to movement backward, because the second pair of legs slopes backward at an angle of about 35°, the third pair also slopes backward at an angle of about 65°, the fourth pair remains almost vertical and the fifth pair of legs slopes forward at an angle of about 70°. This movement resulting from the more effectively braced second and third pair of legs opposing the fifth pair of legs is too irregular to produce a dynamic orientation.

The responses of the fiddler crabs to the neurophil drugs used may be compared with the results with other animals obtained by other investigators. The solutions were made by dissolving the alkaloid in 200 cc. of sea water in a finger bowl, which amount was sufficient to cover the animals. The control animals were placed in the same amount of sea water. Two animals were placed in each solution and from 2 to 5 separate experiments were performed on different days. Unless otherwise specified the solutions were all of 1 : 1,000 strength. During the time of observation the animal was placed in a large crystallizing dish containing an even layer of moist sand, after which it was returned to the solution.

Atropine $SO_4$ (1 : 500 to 1 : 10,000), apomorphine (sat. sol.), morphine $SO_4$, caffeine, codeine and digitonin were without any visible effect on the animals during 40 hours' exposure.

When an object is brought near a control *Uca* placed in the observation dish, the animal raises its large cheliped above its head, toward the stimulating object, and backs away from it. This response is inhibited after 5 min. immersion in picrotoxin (sat. sol.), 10 min. in strychnine $SO_4$, 15 min. in phenol or pilocarpine HCl and somewhat later in nicotine. Even when vigorously stimulated the animal cannot raise the chela. Conversely this response is greatly exaggerated after 3–4 minutes in camphor; the animal now does not back away but will grab a pencil or other object in its big claw and will hold it more firmly, and for a longer time than the control animal can be induced to do after the control animal has been "cornered" at the edge of the dish.

Picrotoxin, phenol, and veratrine (sat. sol. alkaloid) reverse the usual activity of the legs so that the legs on the opposite sides of the body pull against each other rather than moving together, as has been mentioned. An analysis at the locus of this effect might perhaps be made with the aid of a galvanic current as has been done with *Lineus*.[1]

All of the legs except the chelipeds are drawn upward and toward the body by contraction of the dorsal musculature of the legs (opisthotonic) after 1/2 hour's immersion in strychnine $SO_4$, phenol or camphor. The strychninized animal's body rests in the sand when the legs are drawn up but the animal can move on stimulation in any direction. This would seem to result from a partial paralysis of parts of the neuromuscular system rather than a specific effect on synapses. Nicotine, veratrine and pilocarpine HCl cause the legs to be extended. In nicotine and in picrotoxin the legs are first extended and later those of the opposite sides are drawn together, so that the animal instead of being held only a few millimeters from the sand is raised up 8/10 mm. above the substratum. After several hours in phenol the legs are drawn together toward the center of the body but the distal segments of the legs are not extended.

Very little asymmetric behavior of the unequal chelipeds of the animals was observed. The male crabs after veratrinization tend to move sidewise in the direction of the large chela. The

[1] See Crozier[7]; *cf.* also Kropp, B., and W. J. Crozier, 1928. Jour. Gen. Physiol., 12: 111–22.

legs were drawn more closely toward the body on the side opposite the large chela as this phase of the picrotoxin effect appeared. The chief difference noticed was that when this large chela was prevented from being drawn close to the body the animals were unable to right themselves after falling on their backs.

Camphor and to a lesser extent nicotine seem to lower the threshold of excitation and the animals are more active than the control animals. The same drugs also affect the chromatophores, so that the animals become lighter in color and after an hour are a light blue. This coloration persists for some time after apparent death.

The reversal of the response of the large claw by strychnine in *Uca* resembles a similar reversal of the response of the prolegs of caterpillars found by Crozier [2] to result from the injection of atropine. Camphor excites the fiddler crab but inhibits in the grasshopper.[3] The specific effect of camphor in determining the backward swimming of *Crangon* [4] is not parallel with *Uca*. Backward movements of the crayfish are impeded by strychnine,[2] but this drug has no such effect on the fiddler crab. The effect of strychnine and nicotine on the legs of the fiddler crab is comparable to the effect of these drugs reported by Moore,[5] who found that strychnine causes the arms of *Asterias* to bend dorsally while nicotine forces the arms to bend ventrally.

The responses of *Uca* to strychnine, picrotoxin, nicotine, camphor, and phenol, but not to atropine or caffeine, indicate that the crab neuromuscular system is more like that of the insects than like that of the worms.[6, 7] An isopod, *Asellus*, is unaffected by strychnine, caffeine, atropine, and nicotine[8]. The greater alkalinity of sea water may be more favorable to a combination of the alkaloids with the tissue proteins, as has been suggested by the experiments of the Petrunkins,[9] and may

[2] Crozier, W. J., 1922. BIOL. BULL., 43: 239–45.
[3] Crozier, W. J., and G. F. Pilze, 1924. Amer. Jour. Physiol., 69: 41–2.
[4] Moore, A. R., 1917. Proc. Nat. Acad. Sci., 3: 598–602.
[5] Moore, A. R., 1920. Jour. Gen. Physiol., 2: 201–4.
[6] Moore, A. R., 1921. Jour. Gen. Physiol., 4: 29–31.
[7] Crozier, W. J., 1927. Jour. Gen. Physiol., 10: 395–406.
[8] Fries, E. F. B., 1928. Jour. Gen. Physiol., 11: 507–13.
[9] Petrunkin, A., and M. Petrunkin, 1927. Jour. Gen. Physiol., 11: 101–10. *Cf.* also Crozier [2].

account for the different effects on the fresh water and marine arthropods. Since the crab is not affected by caffeine it may have a less differentiated nervous system than the squid.[6]

## Summary.

A reversal of the usual motor responses of the legs of the male fiddler crab, *Uca pugnax*, has been observed when the animals have been in solutions of picrotoxin, strychnine, phenol, philocarpine, or veratrine in sea water. Camphor increases the irritability of the animals. Atropine, apomorphine, morphine, codeine, caffeine, and digitonin are without effect on the crabs. The results of these experiments are discussed in relation to those made with other arthropods.

# THE CONDUCTION OF THE NERVOUS IMPULSE THROUGH THE PEDAL GANGLION OF MYTILUS.

OSCAR W. RICHARDS.

*(From the Department of Biology, Clark University, Worcester.)*

The object of these experiments was to determine the change in the rate and nature of nervous conduction through the pedal ganglion of *Mytilus*. The large Pacific Coast mussel, *M. californianus* Conrad, was used in the early experiments which were made at the Hopkins Marine Station of Stanford University during the summer of 1926. The latter experiments were made at the Marine Biological Laboratory during the summer of 1928, using *M. edulis* L. The mussel was used rather than *Ensis* (*cf.* Drew, 1908) because the former permitted an independent nerve muscle preparation of the foot while the cerebral ganglia must be included in a similar preparation of the latter in order to obtain a reflex response. Nerve conduction through the ganglion was much slower with both species of animals than the conduction in the pedal nerve. This difference was greatly reduced after a solution of strychnine was placed on the ganglion. Camphor also removed the inhibiting effect of the ganglion, and atropine caused a reversal of the contractions of the longitudinal and circular muscles of the foot.

## I.

The foot of *M. californianus* obtained by severing it from the animal makes an excellent nerve-muscle preparation. It quickly relaxes from the stimulation of the cut and may be pinned firmly to a wax-bottomed dish by two pins placed about 8 mm. from its distal end. The pedal nerve and ganglion may be easily exposed by a cut through the dermis the length of the foot. After the position of the nerve is familiar it is not necessary to make cuts other than at the points of stimulation.

In the following experiments the nerve was not exposed further

than by carefully pushing the points of the platinum electrodes through the skin, so as not to injure the nerve, until the records were completed. After that the ganglion was exposed to make certain that the points of stimuli were properly located with respect to the ganglion. This procedure permits several determinations with little injury to the animal.

The tip of the foot of the preparation was connected to a light writing lever by means of thread. It was necessary to reduce the speed of the slide myograph by substituting a weight to draw the carriage for the spring that comes with the instrument. The key switch on the instrument made the stimulus and the rate of movement of the paper carriage was shown by recording a 100 d.v. tuning fork. The precautions indicated by Jenkins and Carlson (1903) and Maxwell (1907*b*) for avoiding experimental difficulties were followed in these experiments. The rates of nervous conduction were calculated from measurements of the times of the latent periods.

The average rate of nervous conduction of 64.2 cm. per second was obtained from 16 determinations made with 4 different preparations. The room temperature varied from 18–20° C. The animals were large, about 200 mm. long (*cf.* Richards, 1928), so that the average nerve length was about 25 mm. This length and the small probable error of ± 1.8 cm. per second indicate the reliability of the determinations.

However, if the rate of conduction through the ganglion be determined it is found to be 22 per cent. ± 2 per cent. slower than conduction along the nerve trunk distal to the ganglion. This strongly suggests the possibility that Fick[1] may have stimulated through a ganglion when in 1863 he found for the "nerve conduction" of *Anodon* the surprisingly low rate of 1 cm. per second. This rate is much slower than the rates for other molluscs are reported to be (*cf.* Jenkins and Carlson, 1903, and Rogers, 1927).

## II.

The smaller size of the Eastern mussel, *M. edulis*, necessitated some modification of the technique. The shell was carefully opened by cutting the posterior retractor muscle and the pedal

[1] Quoted by Jenkins and Carlson (1903).

ganglion exposed by breaking through the tissue along the anterior retractor muscles of the foot. The small pedal ganglion is bright orange (Field, 1922), which aids in finding it. The animal was held in a small dish and then the tip of the foot was connected with a writing lever. By using the animal in the shell in this manner a less injured preparation is obtained. As the foot was too small to anchor near its tip, the rates had to be calculated from the latent periods of the reflex time from stimulating the foot distally and close to the body. As the contraction always occurs first at the base of the foot these times may be directly compared. A few determinations with the isolated foot gave essentially the same conduction rates, with a greater variability which was due to the technical difficulties involved with so small a preparation.

The average rate of conduction was found to be 92.9 cm. per second, from 12 determinations with 8 animals. Because the animals were smaller, the difference between the points of stimulation averaging about 18 mm., the probable error is greater, being 3.3 cm. per second. The room temperature averaged 24° C. and when this higher temperature in taken into consideration we see the rate in the two species of mussels is about the same.

The slowing of the conduction rate through the ganglion was determined by stimulating the nerves entering the ganglion, and the pedal nerve, stimulating in both cases close to the ganglion, and noting the differences in the latent periods of the responses. The distance between the points of stimulation averaged about 3 mm. The average time of conduction through the ganglion was 0.0087 sec. from 9 determinations with 7 different animals. This is about 2.7 times slower than the conduction along a nerve trunk would be, and further supports the suggestion of the possible cause of the slow rate found by Fick mentioned in the last section.

## III.

If this delay in the rate of conduction through the ganglion is due to a synaptic resistance, it might be expected to be reduced when the ganglion is treated with strychnine. This proved to be the case, as five minutes after applying 1 : 500 strychnine $SO_4$

in sea water to the outside of the ganglion the difference dropped to an average of 0.003 second. This average was obtained with 6 tests with 4 animals. Before strychninization the average delay for the same animals was 0.008 second.

Strychnine does not "open the synapses" in the same way as with vertebrates, because unless the animal is stimulated the foot shows no contraction when its actions are recorded on a kymograph (Fig. 1). The threshold of excitation of skin, nerve

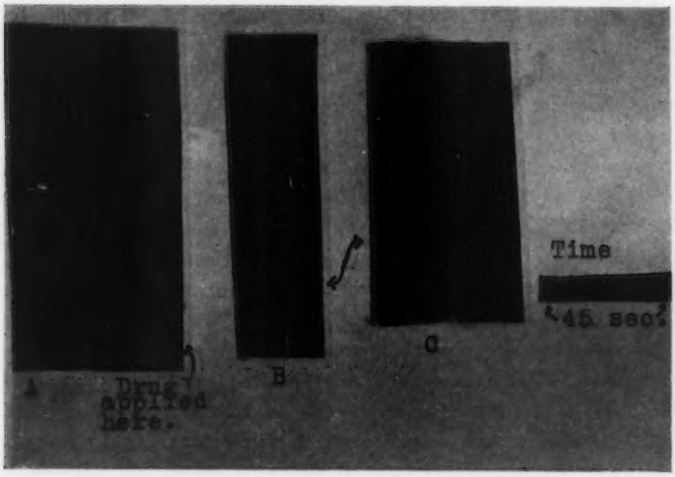


FIG. 1. The effect of applying strychnine to the pedal ganglion of *Mytilus*, as seen in the contraction of the foot. *A*, contraction before using the drug; *B*, contraction 6 minutes later; *C*, contraction 12 minutes later, which shows decreased irritability of the preparation.

and ganglion is greatly increased. Ten minutes after applying the drug the skin is barely sensitive to touch. If the foot is free to move and not attached to the recording apparatus, writhing movements are seen as soon as the drug is applied to the ganglion. These soon cease and the foot remains partly contracted. The circular muscles are contracted proportionally more than the longitudinal muscles. After an hour the foot becomes normally relaxed and quickly responds to stimuli.

Picrotoxin and philocarpine HCl (1 : 200) seem to have no

effect on the ganglion. Phenol 1 : 200 keeps the foot about half contracted, but its irritability remains the same as that of the control animal whose ganglion was washed with sea water. After the application of creatin 1 : 200 to the ganglion the circular muscles at the base of the foot remained contracted and prevented the foot from completely shortening when it was stimulated. The preparation seemed about as irritable as the controls. Thirty minutes after the application of caffeine 1 : 200 the foot became contracted to about one fourth of its normal size and exhibited rapid spontaneous movements. This may be due to some other and secondary effect rather than to the drug itself (*cf.* Maxwell, 1907*a*). Nicotine 1 : 200 may have reduced the sensitivity of the preparation slightly, but this was barely apparent.

Immediate squirming of the foot is seen when 1 : 200 atropine $SO_4$ is placed on the pedal ganglion. Two minutes later the foot is sensitive to stimuli but it can only contract half-way as the circular muscles at the base of the foot are tightly contracted and the tip of the foot remains flabby and relaxed. The foot becomes hypersensitive to stimuli, partially contracts and relaxes quickly, then a control foot seven minutes after the drug was applied. When it is relaxed the base is narrow from the contraction of the circular muscles, but the rest is greatly elongated as if the longitudinal muscles were forcibly relaxed. This seems to be a reversal of reciprocally acting muscles and somewhat resembles the "reversal" observed after atropine is injected into the body of caterpillars (Crozier, 1922). The effect of atropine on the mussel is similar to the effect of strychnine on the foot of *Chromodoris* (Crozier & Arey, 1919). It is very difficult to record the effect of atropine graphically as the increasing relaxation of the foot necessitates frequent readjustment of the base line and the slight pull on the foot of the writing lever initiates frequent movements of the foot. Such movements were rarely observed with the control preparations.

When a few drops of saturated solution of camphor in sea water were dropped on the pedal ganglion the foot immediately contracted and quickly relaxed. Within two minutes the threshold is so lowered that a jar of the table initiates a spasmodic con-

traction of the foot and adjacent musculature. Soon spasmodic movements of the foot are observed without any apparent stimuli. Six minutes later mantle spasms are observed. The foot becomes so irritable that it immediately contracts when it relaxes enough to barely touch the mantle or a gill. These twitchings are illustrated in Fig. 2. If the ganglion be isolated

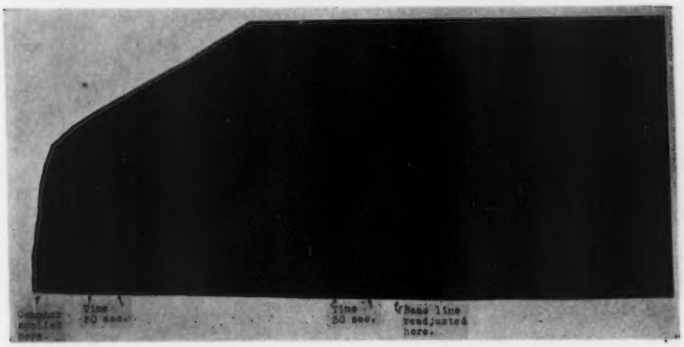


FIG. 2. The effect of applying camphor to the pedal ganglion of *Mytilus*. (*Cf.* Fig. 3.)

by cutting the collaterals which connect it with the rest of the nervous system, the foot relaxes and remains relaxed unless stimulated (Fig. 3). Twenty minutes after camphor is applied, the foot is fully contracted to about one quarter of its usual size. Both the circular and longitudinal muscles are then tightly contracted. This effect of camphor resembles the effect of strychnine in lessening synaptic resistance in certain other animals.

The slower rate of conduction of the nervous impulse through the pedal ganglion of the mussel is abolished five minutes after the ganglion is bathed with strychnine, and the irritability of the foot neuromuscular mechanism is reduced. Atropine causes a reverse of the reciprocally acting muscles of the foot. Camphor seems to open the pathways so that an almost continuous discharge of nervous impulses keeps the foot in active movement until the foot is so contracted that it can hardly move. That this is not due to a stimulation of the ganglion cells is shown by

the relaxation and inactivity of the foot when the pedal ganglion is isolated from the rest of the nervous system.

The supra-œsophageal, pedal, and (in cephalopods) the stellate ganglia respond specifically to the application of strychnine or phenol (*cf.* Baglioni, 1905, Frölich, 1910, Moore, 1917). The injection of strychnine into *Limax* suppresses the phototropic

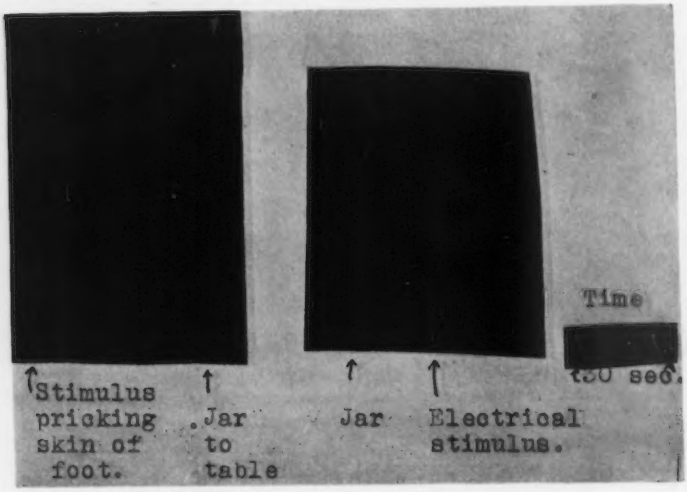


FIG. 3. The effect of camphor on the pedal ganglion of *Mytilus*, as seen in the contraction of the foot. The foot shows contraction only after direct stimulation of the preparation, when the cerebro-pedal connectives have been severed to isolate the pedal ganglion and nerve from the rest of the nervous system. (*Cf.* Fig. 2.)

circus movements of the animal without affecting the activity of the foot. Crozier and Federighi (1924) consider that this result is due to central "competition" between impulses, resulting in the release of pedal waves and in the maintenance of a turning posture.

Strychnine and atropine seem to affect different parts of the organization of the pedal ganglion of *Mytilus* and camphor seems to affect most of the neurones in the ganglion. Strychnine may do the same, but the effect may be masked by some direct or secondary anæsthetic action. These observations intimate caution in the interpretation of the possible locus of the strychnine

effect as synaptic. The chemical differences among the drugs used in this study imply chemical differences in the combination of the drugs with the nervous elements, but enough information is not yet available for a classification of the differences. The cephalopod is sensitive to caffeine, atropine and camphor (Moore, 1917). *Mytilus* is poisoned readily by atropine and camphor and less so by caffeine. The effect of strychnine is less pronounced on *Mytilus* than on *Chromodoris* (Crozier and Arey, 1919). The effect of drugs would place the mussel between the nudibranchs and the cephalopods, which is in agreement with taxonomy.

## SUMMARY.

The rate of nervous conduction in *Mytilus californianus* was found to be 64.2 ± 1.8 cm. per second at 18–22° C. and that of *M. edulis* was found to be 92.9 ± 3.3 cm. per second at 24° C. The rate of conduction through the pedal ganglion was much slower in both animals. Treatment of the ganglion by bathing it with strychnine abolished this delay. Applying camphor to the ganglion results in the foot exhibiting almost continuous movement with no apparent source of stimulation. These movements stop if the pedal ganglion is isolated by cutting the connectives just anterior to the ganglion. The longitudinal muscles of the foot are greatly relaxed and the circular muscles, especially at the base of the foot, contract when the foot is stimulated after applying atropine to the ganglion. This is just the reverse of the response of the foot of the control animal to stimulation. These observations are discussed with respect to the drug effects with other animals and to the use of strychnine as a test for the function of the synapse.

## BIBLIOGRAPHY.

**Baglioni, S.**
'05 Z. allg. Physiol., 5: 43.
**Carlson, A. J.**
'05 Am. Jour. Physiol., 13: 351.
'11 Am. Jour. Physiol., 27: 323–30.
**Crozier, W. J.**
'22 BIOL. BULL., 43: 239–45.
**Crozier, W. J., and L. B. Arey.**
'19 Jour. Exp. Zoöl., 32: 261–310.
**Crozier, W. J., and H. Federighi.**

'24 Jour. Gen. Physiol., 7: 221–4.
**Drew, G. A.**
'08 Jour. Exp. Zoöl., 5: 311–26.
**Field, I. A.**
'22 Bull. U. S. Bureau Fisheries, 38: 127–259.
**Frölich, F. W.**
'10 Z. allg. Physiol., 11: 269.
**Jenkins, O. P., and A. J. Carlson.**
'03 Am. Jour. Physiol., 8: 251–69.
**Maxwell, S. S.**
'07*a* Jour. Biol. Chem., 3: 21–4.
'07*b* Jour. Biol. Chem., 3: 359–85.
**Moore, A. R.**
'17 Proc. Nat. Acad. Sci., 3: 598–602.
'21 Jour. Gen. Physiol., 4: 29–31.
**Richards, O. W.**
'28 The Nautilus, 41: 99–101.
**Rogers, C. G.**
'27 Textbook of Comparative Physiology, New York, 537 pp.

# GROWTH OF A POND SNAIL, *LYMNÆA STAGNALIS APPRESSA*, AS INDICATED BY INCREASE IN SHELL-SIZE.

EDWARD D. CRABB,

ZOÖLOGICAL LABORATORY, UNIVERSITY OF PENNSYLVANIA.

This paper is a result of attempts to develop a method of rearing pond snails in the laboratory for experimental purposes in which early development of healthy animals that lay a quantity of normal viable eggs at all seasons of the year was of prime importance. After this point had been attained various conditions of food and media were tried to see whether the results would confirm those of other workers who have investigated growth in fresh-water pulmonates or help to explain their conclusion. Since practically no literature on growth in *L. s. appressa* is available, it is necessary to turn to the work on the old world species for comparison. After comparing shells of my stock and of wild individuals from the same lake (in Michigan) which afforded the progenitors of my experimental animals with specimens of *Lymnæa stagnalis* of the P. Hesse and other collections in The Academy of Natural Sciences of Philadelphia, which were collected in many parts of Europe, I am satisfied that there is no constant difference between the American *L. stagnalis appressa* and the European *L. stagnalis* with regard to shell-form and size. British shells of *L. stagnalis* measure 47 × 21 mm. (Hogg, 1854, Fig. 7) and 34–50 × 18–28 mm. (Ellis, 1926), but the radula differs somewhat in American and in European snails (F. C. Baker, 1911). Thus it follows that the age and growth relation of the shell in *L. s. appressa* Say should be very similar to that of the European species, *Lymnæa stagnalis* Lin.

An idea which prevails among some of the conchologists is that in nature the volume of the medium largely determines the maximum size attained by the snails so that individuals of the same species grow larger in large bodies of water than in small, and this differential growth is offered as a means of explaining

the origin of races, or subspecies. Others attribute differences in shell-size to transient, imposed conditions which play no part in the origin of races. The genetics of pond snails has been considered in a previous publication (Crabb, 1927*a*). Semper (1883) indicates that Hogg, Blanchard, and he were probably the first investigators to demonstrate by laboratory experiments that variation in shell-size in *Lymnæa stagnalis* is dependent upon the size of the body of water in which the snail lives. Hogg (1854: 103) was confident that he had arrested growth in *L. stagnalis* by keeping the snails in small vessels, and Semper (1874, 1883) maintains that increase in growth is directly related to the volume of medium up to 4–5 liters per snail, but that additional increase in volume has no further effect on growth. Apparently he was confident of evolutionary significance of his results, for he stated (1883: 163) that every *Lymnæa* which failed to attain a length of 20 mm. during the first year of its life under unfavorable conditions produces a dwarf race if the conditions which have retarded its growth be repeated during the following years. He explains the relation of growth to volume (1883: 166) by postulating the theory that there is present in water a minute quantity of an unknown substance which is probably absorbed through the skin, the amount of which that can be absorbed "in a given time depends upon the volume of the water and increases or diminishes with it, . . . ." Shells of the third and fourth generations of *Lymnæa megasoma* reared in the laboratory had more slender spires and differed materially in other ways from the wild Vermont parent and led Whitefield (1882) to suppose that he had created a new form, presumably by rearing individuals in a smaller volume of water than their parents were accustomed to in nature. *Lymnæa traskii* living in a large glacier-fed lake in the Canadian Rockies averaged 27½–60½ per cent. smaller than those living in a near-by pond having a higher maximum temperature and more abundant food supply (Mozely, 1928).

Most aquariasts and investigators who have reared fresh water pulmonates agree that these snails do best in a relatively large volume (about a liter or more to the snail) of water (Whitefield, 1882; Semper, 1874–1883; de Varigny, 1894; Willem, 1896;

Legendre, 1907, 1908; F. C. Baker, 1911; Colton, 1912, and Popovici-Baznosanu, 1921, with *Lymnæa*). It has been shown in other aquatic forms that reduced volume, including crowding, reduces the growth rate of the animals, a part of the body, or the rate of division in one-celled organisms (Kulagin, 1899; Woodruff, 1911, 1913; Robertson, 1923, and Greenleaf, 1926, on Infusoria; Vernon, 1895, 1899, 1903, plutei; Warren, 1900, Daphnia, and Young, 1885, tadpoles). Most of these workers appear to believe that this retardation of growth is the result of foul media and, or, insufficient food, but none of them has shown conclusively just what the cause is. Robertson (1923: 139) maintains that in Infusoria, although growth (as indicated by multiplication) is retarded by old culture media, "the origin of the retardation . . . may readily be shown to reside neither in exhaustion of available foodstuffs nor in the accumulation of toxic products within the medium." To me the results of his experiments indicate a "specificity of toxicity" which is Woodruff's (1913) explanation of the results of his experiments in which Infusoria grew readily in old Hypotricha media, but were markedly retarded when cultured in old Infusoria media. A similar "specific fouling injury" was found to exist in Daphnia cultures (Vernon, 1899, 1903). However, he shows that old cultures are more favorable to hatching and growth in a different than in the same species of Echinoid.

Besides Semper's novel explanation of the cause of stunted growth there are others that pertain to fresh-water snails. Willem (1896) put from 1 to 10 *Lymnæa ovata* and 1 to 5 *Planorbis corneus* in equal volumes of media with food, light, temperature, etc., the same and by aërating "to saturation" he got a maximum of 37.32 per cent. increase in growth in six snails over an equal number which were not aërated. He concludes that oxygen is the factor which determines growth and states "that in the basommatophores cutaneous respiration is more important than pulmonary respiration and that it alone is sufficient for the animal" (p. 562). De Varigny (1894) thinks that growth in *Lymnæa* is directly related to increased surface area, even though the volume is reduced, by permitting the snail to take an increased amount of exercise, for dwarfing from crowding is not

due so much to the actual numbers in the vessel as to the "moral influence of numbers which inhibit exercising, just as a man does not like to take a walk on a crowded street. . . ." Popovici-

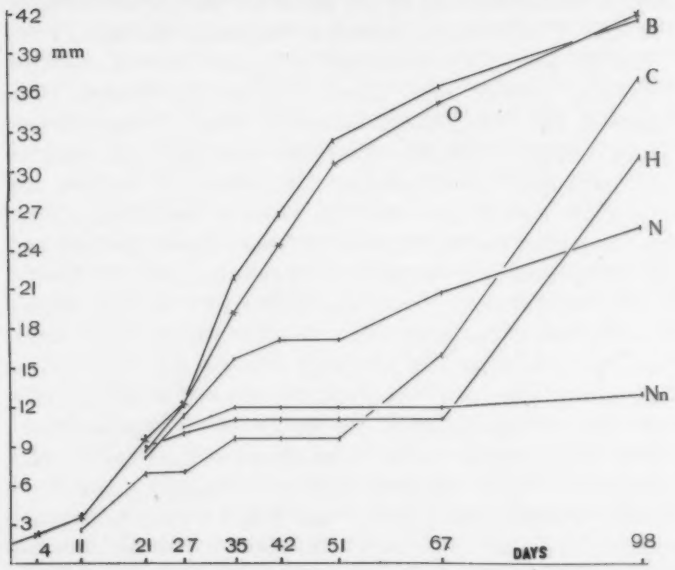


FIG. 1 shows the effect of foul medium on growth of the shell. The individuals are all from the same mass of eggs and were isolated in tap water at or before hatching. All conditions, light, temperature, etc., except media, were kept the same in each case. Food: leaf lettuce, boiled wheat and filter paper every two or three days. *B*, mean length of 15 snails; medium was poured out and fresh supplied every 7 days. The deviation from the mean is − 4 at age of 51 days. *C*, length of the smallest individual in the *O* group. *H*, length of the smallest individual of the *B* group (not included in *B*). *N*, mean length of 14 snails, except that three died before the last measurement; therefore only 11 are represented in it. Excrement never removed but allowed to decay; fresh tap water added to maintain the standard volume. The probable error of the mean at the age of 51 days was ± 1.18; at age 98, ± 1.12 mm. *Nn*, length of the smallest individual of the *N* group. *O*, control (elsewhere designated as "Master Control") mean length of 15 snails; tap water; excrement siphoned off 3 times each week, all the water poured off as often as it became foul and fresh added. The probable error of the mean length at the age of 51 days was ± 0.635; at age of 98, ± 0.711.

Baznosanu (1921) holds that food is the most important factor in producing growth in pond snails, and that the effect of excrement is not as important as Legendre thinks. However, Legendre

(1907, 1908) found that clean cultures favor the growth of both snails and water plants, while excrement, "especially the liquid or soluble kind," retards growth. He (1907) records five experiments with *Lymnæa stagnalis* in which volume, temperature, light, surface and food were the same and in which the water on the controls was changed every two days while that on the experimental animals was not changed, only fresh water added to maintain the volume. By this treatment he obtained a maximum length in control animals of 25 mm. at the age of 97 days. Further analysis of his results shows that his differences in the mass cultures are insignificant (maximum 1.8 mm.) when the relative numbers in each experiment and in the control are considered and that the maximum growth-rate of his controls, 8.4 mm. in about 74 days (relatively less than half that of my experimental animals (Fig. 1, *N*), indicates that these snails, as well as the experimental animals, were suffering from severe inanition. For the same reason the maximum adult length obtained by Popovici-Baznosanu (1921: 52) in his control *Lymnæa stagnalis* was 26 mm. in 143 days. That the snails in this experiment were started out properly is shown by their length at the age of 29 days, 19 mm., which compares favorably with my best results (Fig. 1, *B*, *O*). Hoffmann (1927), citing Kunkel, 1916, gives the size of a *Lymnæa stagnalis* as 20 × 9 mm. at nine months old and at the age of 20 months, 47 × 23 mm. Legendre (1907), using 1,300 ml. of water to each control culture of ten *L. stagnalis*, obtained maximum lengths of 18–22 mm. in about eleven months. In comparison with this and other work done on the effects of crowding may be mentioned one of my cultures in which eight snails were hatched and reared in a 100 ml. wide-mouthed bottle (ca. 12.5 ml. to each snail), and at the age of 151 days the three smallest averaged 26 mm. in length. Similar results obtained by various investigators rearing freshwater mussels in crates and observed by them in nature have been attributed to crowding and to insufficient food supply (Coker, Shira, Clark and Howard, 1922; Iseley, 1914). Abnormally low growth rates for control animals is a criticism which is applicable to the work on *Lymnæa stagnalis* by the investigators cited in this paper. Most of these investigators

apparently depended upon Lemna, Algæ, Chara, Elodea and other water plants to furnish the major part of the food for their snails. Popovici-Baznosanu (1921) speaks favorably of lettuce for snail food, but adds that "of all foods microflora is the most important."

In general my growth curves are the sigmoid type and agree with Semper's (1883: 163), and Roberts' (1923) curves and his

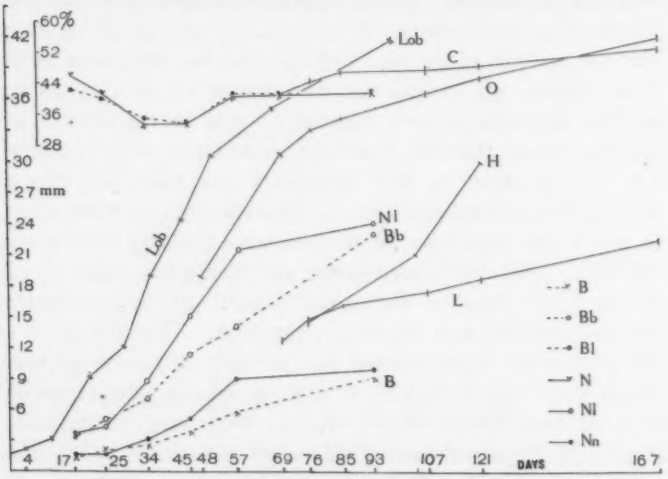


FIG. 2 shows effects of food, medium and crowding. Each individual, except group *H*, was reared in isolation in tap water under the same temperature and light conditions. *B*, mean width; *Bb*, mean length; *Bl*, mean index of stature of 7 individuals which were fed lettuce, hard-boiled eggs and filter paper until 57 days old; then fed leaf lettuce only; excrement removed once every 7 days. A mixed culture of microörganisms was included in the food the first two times the snails were fed. *N*, mean index of stature; *Nl*, mean length; *Mn*, mean width of 8 snails fed leaf lettuce and filter paper the first 25 days and afterwards a diet of boiled wheat, baked potato and leaf lettuce until 57 days old when they were given leaf lettuce only and excrement removed every 7 days. *C*, mean index of stature; *L*, mean width; *O*, mean length of 10 individuals, each of which was reared in solation from hatching and kept on a diet of leaf lettuce, filter paper, and occasionally boiled wheat was added and excrement removed two to three times each week. *H*, mean length of 6 larger individuals from an over-crowded mass culture which were placed in an aquarium containing about 7 litres of tap water and fed leaf lettuce and filter paper. *L*, see *C*. *Lob*, master control (mean length of 15 individuals, Fig. 1, *O*). *O*, see *C*. Snails represented by graphs *B*, *Bb*, *Bl*, *N*, *Nl*, and *Nn* are from one egg mass; *C*, *H*, *L* and *O*, from another, and *Lob* from a third mass of eggs.

idea of three "cycles" in the growth of an individual to the extent that there is an initial period of relatively slow growth followed by a period of relatively rapid growth which is succeeded by a slowing up in the growth of the anterior margin and an increase in growth of the lateral margin of the shell. In *Lymnæa* this last "cycle" continues until death. These changes in length are attended by changes in the relative width of the shell (Fig. 2, *Bl*, *N*).

It is to be regretted that circumstances prevented rearing larger numbers of individuals in certain of the following experiments, as well as having made it impractical for me to supplement them adequately.

## Material and Methods.

The original stock for these experiments was obtained from Third Sister Lake, near Ann Arbor, Michigan. The animals used in this work were free from trematode infection, thus obviating a factor, common to wild snails, which Hurst (1927) shows markedly affects vital activities in *Physa occidentalis;* neither were they retarded in growth or age of maturity by low temperatures, such as Weymouth, McMillin and Holmes (1926) have clearly shown is the case in the Pacific razor clam, *Siliqua patula*, along the coast from Washington to Cordova, Alaska. In order to eliminate, as far as possible, factors which might arise from genetic or extrinsic causes controls were usually selected from the same mass of eggs as the experimental animals and, whenever practical, the graph of the "master control," which is the mean length of 15 individuals (Fig. 1, *O*), is shown in each chart to facilitate comparisons.

The size of the shell, instead of the weight of the animal, is used to determine growth in this snail because adult individuals are capable of retaining quantities of water varying to more than a cubic centimeter.

The quantity of medium in the various cultures was maintained by replacing that lost in siphoning out excrement or by evaporation when the snails were fed, unless otherwise stated. In the isolation cultures, unless otherwise explained, a single egg or a single snail was placed in a clear glass fingerbowl of about 200 ml.

capacity, but containing 150–175 ml. of medium, and covered with a clear glass plate to prevent the escape and death by desiccation of the snail as well as to inhibit evaporation of the media.

By means of a room thermostat water in the various cultures was kept at approximately 18–20° C. throughout the year, except during a few successive days of very warm weather during the summer of 1925. The temperature of the tap water was raised to the desired degree before subjecting the snails to it.

Unless otherwise stated, the snails were never without food, a sufficient quantity being given to them to last until the next feeding time, when the remaining food was removed and fresh supplied.

Aëration was accomplished by allowing air from the compressed air system to bubble through the water in which the snails lived.

Direct sunlight was applied by setting the uncovered finger-bowls on the broad ledge outside the window.

The measurements are all in millimeters and represent the maximum length, or width, of the shell, with the aperture turned down as in the crawling posture, taken with fine-pointed dividers and recorded to the half millimeter. Very small individuals were placed on a Carl Zeiss Objectmikrometer, all free water removed with a piece of filter paper to eliminate refraction, and the measurements read to one fifth of a millimeter with a Leitz stereomagnifier using 7× oculars. After the width was recorded it was necessary to turn the snail onto its back and orient the shell with needles so that the true length could be obtained. This tedious procedure was made necessary by the fact that in the young shell the plane of the aperture lacks several degrees of coinciding with that of the apex; therefore, when placed on its ventral surface this factor and that of the protruding body whorl and exuding mucous fluid caused the shell to lie in such a position that the observed length is much less than the actual caliper length. The comparatively dry dorsal surface of the shell adheres sufficiently to the moist glass rule to facilitate orienting it. These two axes more nearly coincide in the larger shells, and too one's ability to orient the

shell and measure it with dividers obviates the difficulties encountered in obtaining the length in young individuals. The index of stature is the width times 100 divided by the length and is graphed to show the relation of width to length in terms of percentage. A total of 136 isolation and 177 mass-reared snails survived and were used in these experiments.

The writer wishes to take this opportunity to thank the Department of Zoölogy of the University of Michigan for having generously supplied the laboratory facilities and equipment for the experiments done from December, 1924, to May, 1926; Professor George R. La Rue and Dr. A. E. Woodhead for supplying stock material from Michigan, and Edward Vanatta, Assistant Curator in the Academy of Natural Sciences of Philadelphia, for kindly showing him specimens and data of *Lymnæa stagnalis*. Without the assistance of Ruby M. Crabb this paper would not have been undertaken.

## Optimum Culture Conditions.

There are indications that in nature this snail does not develop sufficiently the first year to reproduce. However, numerous masses of viable eggs found as late as the last week in October may have been laid by snails hatched during the preceding May. "The duration of life in the family Lymnæidæ is from three to four years, full maturity being reached in about two years" (F. C. Baker, 1911). My *L. stagnalis appressa*, reared under standard isolation conditions, reached maturity within three months and few of them lived longer than nine months; due, no doubt, to the fact that since the snails had no rest-periods, such as are brought about by seasonal conditions in nature, the vital processes continued actively with the result that their life span was reduced to a few months.

Although my records are not sufficiently complete to establish the minimum age at which isolated individuals of various growth-rates may be expected to begin laying, they do show that in all cases individuals reared under optimum conditions lay more eggs per week, a total of many more eggs during life, and that these eggs have a much higher percentage of viability than was the case in individuals reared under conditions that did not result

in optimum growth-rate. The percentage of viable eggs appears to be related to the direct effect of the medium upon the eggs after laying rather than indirectly through affecting the mother during development of the eggs. The growth-rates represented by Fig. 1 bring out this point well. Eggs laid by the fourteen individuals of the *N*-group were worthless for experimental purposes because of the very high death rate which increased as the snails grew older and the medium became more unfavorable for hatching. In this case it was clear that high mortality of the embryos and low growth-rate of the mothers were correlated. The specific effects of the medium were not investigated. In groups *B* and *O* the growth-rate is nearly the same, the difference being in favor of *B* between the ages of 27 and 88 days, but the eggs of *O* had a higher percentage of viability than those of *B*. From the point of early egg production, it seems that the *O* group is inferior to either of the other two groups. In group *N* (foul culture) the first individual laid at the age of 70, the second at 88, and the third at 118 days. In the *B*-group the first five snails laid at ages of 85–90 days while in the *O*-group only two individuals had laid at the age of 85–90 days. Soon, in quantity as well as quality, the eggs of the *O*-group surpassed those of the other two groups. Therefore the *O*-group was used as a "master control" for all the experiments. Three snails isolated before hatching and reared in fingerbowls at this laboratory under conditions as nearly like those used at Michigan as was possible had the following measurements when 172 days old: 40 × 20, 42 × 21 and 44 × 21 mm.

## Effect of Food on Growth.

Several experiments were undertaken with the idea of determining the effect of different foods on growth-rate. That boiled wheat is superior to hard boiled eggs and random microörganisms is indicated by the difference between *Nl* and *B*, Fig. 2 and also by the fact that *Nl*, Fig. 2, failed to keep pace with *B*, or *O*, Fig. 1. Although there is a paucity of data recorded (Fig. 7, *B*, *C*; 8, *N*) iceberg lettuce appears to be far inferior to leaf lettuce, especially for young snails; large snails do much better on the green outside leaves than on the more tender white leaves.

Whether or not this is the result of a higher chlorophyl and vitamine content has not been proven. It was found that when large snails were fed an abundance of leaf lettuce, iceberg lettuce, green cabbage leaves and *Elodea* they ate the leaf lettuce, rejected the others and laid their eggs on the green cabbage (Crabb, 1927) and *Elodea*. When the leaf lettuce was not replenished they ate the iceberg and continued to lay on the cabbage and *Elodea*. When green cabbage alone was supplied they subsisted upon it, but thin slices of sugar beet, carrot and spinach leaves were eaten more readily than cabbage, and Elodea was very seldom eaten. Incidental food stuffs range from the flesh of their own kind to many plants, for this snail will try its "teeth" on anything it may chance upon, especially if it is something unusual, and usually swallows whatever may get into its mouth.

Desiccated, pulverized endocrin glands (Armour & Co.) were mixed with flour and water to the consistency of bread dough, rolled thin, marked off into equal squares, dried and fed in addition to leaf lettuce. Three snails (Fig. 6, *H*) received anterior lobe of the hypophysis, two, thymus (Fig. 6, *N*) and three others thyroid (Fig. 6, *O*). Since in each case the "gland-bread" began to putrify in a few hours, and since in all three groups growth was retarded to so nearly the same extent, it was concluded that putrefactive products acting on the media, rather than hormones, were responsible for the stunted, malformed shells (Fig. 9, Nos. 19–25).

### Effects of Media, Temperature and Light.

The popular belief that snails live in water having a relatively low calcium content led us to attempt to rear individuals in media of distilled water and cistern water so that the food would be the only source of calcium. In every instance snails reared in calcium-free water failed to grow normally (Fig. 4, *C*, *H;* 5; 6, *L*, *E* and 9, Nos. 1–7). To test the effect of calcium in the water five sets of three individuals each were isolated when one week old in molar solutions of $CaCl_2$ ranging from $m/100$ to $m/10{,}000$. One individual lived to be 71 days old and attained a length of 8.5 mm. and another, 14 days and 3.25 mm. in $m/100$ solution. Two others in $m/10{,}000$ solution lived 34 days, length 8.25 mm.

and 9 days, length 3.25 respectively. Since the control consisting of 6 groups of three each from the same mass of eggs all died within three weeks, and other individuals, mentioned above, lived in calcium free media 155 days, the calcium experiments have no marked significance. However, deVorges (1903) maintains that a bivalve, *Anadontia cygnea*, obtained calcium from the water alone.

Temperature and light are undoubtedly factors in the growth and reproduction of this snail, but our preliminary work does not indicate the quantity or the quality which might be tolerated. Two individuals (Fig. 8, *Nn*) reared in isolation in complete darkness at 18° C. did not make the growth of the master (*E*, Fig. 8) or any of the isolation controls. Four others (Fig. 8, *N*) reared under the same conditions, except for diet, and from the same egg mass did not attain the rate of growth of *B*, Fig. 7, which were fed about the same food, but reared in more light and at two degrees higher temperature. A snail which was isolated at hatching in tap water and fed leaf lettuce was kept in a room in which the temperature remained about 15° C. until 113 days old when it had a length of 7.50 mm.; from this time on the individual was kept in another room at about 20° C. and given the usual care and when 151 days old its length was 8 mm.; 165, 24 × 9 mm.; 179, 30 mm.; 201, 35 mm.; 242, 38 × 18 mm.

Direct sunlight does not appear to increase the growth rate, for three isolated individuals which were thus treated one to three hours daily and fed the standard diet (Fig. 8, *B*) grew no faster than the master control (Fig. 8, *E*) and only one of the three made as rapid growth as the single living isolation control which received an equal amount of sunshine through a plate glass window. Six individuals were treated as in *B*, Fig. 8, except that excrement was not removed and three of the snails were not exposed to direct sunshine. Since the mean length of each group of three did not vary as much as one half millimeter at any measurement, both groups were graphed as *Bl*, Fig. 8.

### Effects of Crowding and Pollution.

Mass cultures have a retarding effect on all individuals and give variable growth-rates usually resulting in "dwarfs" and

"giants" in each culture. The results of the following experiments show that when such individuals are transferred to favorable conditions they may reasonably be expected to rapidly reach the normal, or near normal, adult size. Eight snails were kept in a 100 ml. wide-mouth bottle until 151 days old, when three of the smallest, having a mean length of 26 mm., were isolated under standard conditions. When 165 days old their mean length was 29.75 mm.; 169, 32 mm.; 179, 36.33 mm.;

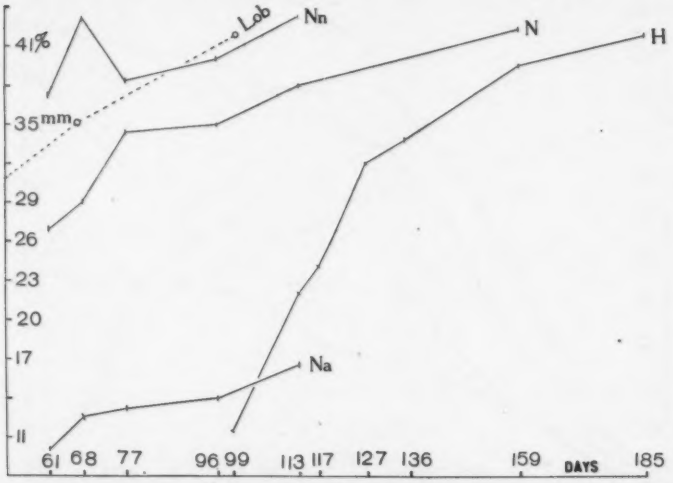


FIG. 3. Crowded compared with isolation cultures. *H*, mean length of the 3 largest and 2 smallest in a mass culture of 24 individuals kept in a fingerbowl until 99 days old then isolated and given standard treatment. *Lob*, master control. *N*, mean length of 6 individuals isolated at time of hatching and fed leaf lettuce, filter paper, an occasional bit of apple peel and boiled wheat; food and water changed twice each week. *Na*, mean width of *N*. *Nn*, index of stature of *N*. All individuals, except *Lob*, are from the same mass of eggs.

188, 37.66 mm.; 201, 39 mm. and 242, 41 mm. Five of the largest of a mass culture of 24 individuals reared in a fingerbowl and isolated when 99 days old had a mean length of 11.50 mm. and attained 42 mm. when 185 days old (Fig. 3, *H*).

Six snails (Fig. 7, 4) reared in a round battery jar containing approximately 150 liters of tap water and fed green lettuce, boiled wheat and filter paper failed to keep up with the control (Fig. 3, *N*) by about five mm., from the 55th to the 185th day.

Two masses of eggs were placed in a battery jar containing approximately 1,500 cc. of tap water and when about 88 days old they were transferred to a round clear glass jar containing about two gallons of tap water. The excrement was siphoned

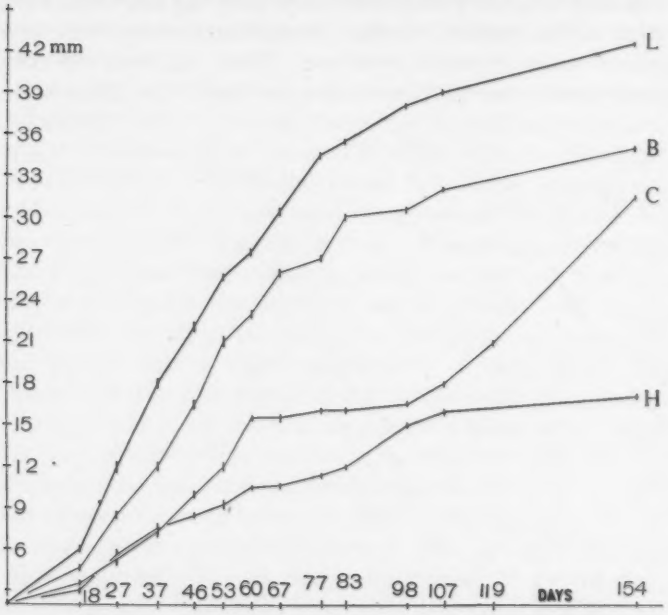


FIG. 4 gives the growth rate as shown by the mean length of the shell in isolated snails fed leaf lettuce and filter paper; all conditions, except those of media, were the same in each case. The snails were fed, excrement siphoned off and fresh medium added three times each week. *B*, three snails, medium, equal parts of distilled and city tap water. *C*, two snails, medium, cistern water until 119 days old when tap water was substituted. *H*, two snails, medium, distilled water. *L*, control, two snails, medium, city water. Although the curve represents the mean length of two individuals, in only one instance, age 107 days, did the measurements of these two vary as much as two mm. All are from the same egg mass.

off and water renewed two or three times each week, the animals fed leaf lettuce and occasionally boiled wheat and filter paper were added to the diet. When about 169 days old they were sorted according to size and placed in three groups to determine their rate of growth. In group I. there were 9 individuals, the smallest measuring 18 mm. in length and 6.5 mm. in breadth,

the largest 25 mm. in length and 11 mm. in width. The mean length and mean width for this group was 23.11 × 9.33 mm. and the mean index of stature was 40.37. In group II. there were 21 individuals which had a minimum and maximum length and breadth of 26 × 11 and 28 × 12.5 mm. respectively with a mean of 26.8 × 11.78 mm. and a mean index of stature of 41.60. In group III. there were 14 individuals which had a minimum

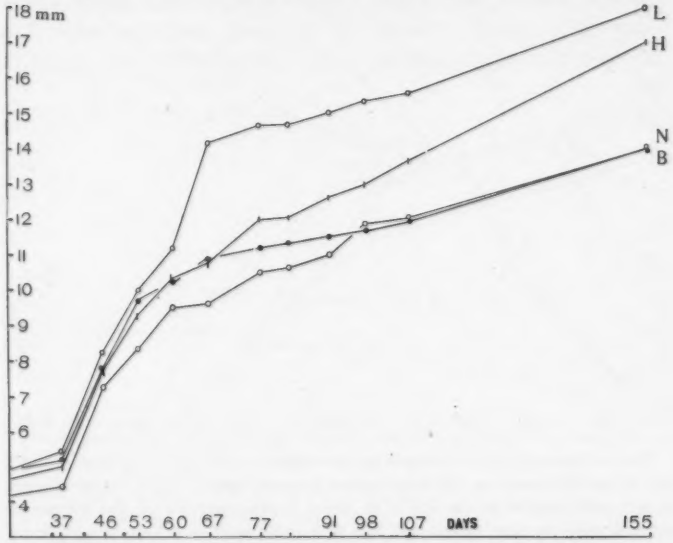


FIG. 5 shows the effect of distilled water. The snails used in this experiment are from the same egg mass as those in Fig. 6 and were reared in a fingerbowl until 26 days old when they were isolated in distilled water, fed leaf lettuce and filter paper, excrement siphoned off and fresh medium added every two or three days. *B*, 3 individuals kept away from the window so that no sunshine could pass through glass to them. *H*, 3 individuals kept near the window so that the sun could shine through glass onto them about 45 minutes each bright day. *L*, 3 individuals, kept away from window with *B*, but aerated 15–20 minutes during the day at least four times each week. *N*, 3 individuals kept near the window with *H* and aerated with *L*. Since the controls died comparison may be made with Fig. 7, *B*, *C*; 8, *N* and with the master control.

and maximum length and breadth of 29 × 12 and 32.5 × 15 mm., a mean of 31 × 13.5 and an index of stature of 43.55. Thus the mean measurements of these 44 individuals at the age of 169 days was 26.97 × 11.53 mm. and index of stature, 41.87.

The length of these 44 snails which spent the last 81 days of their lives in favorable mass culture conditions is about half that of ten individuals (Fig. 2, *C*), reared in isolation from hatching.

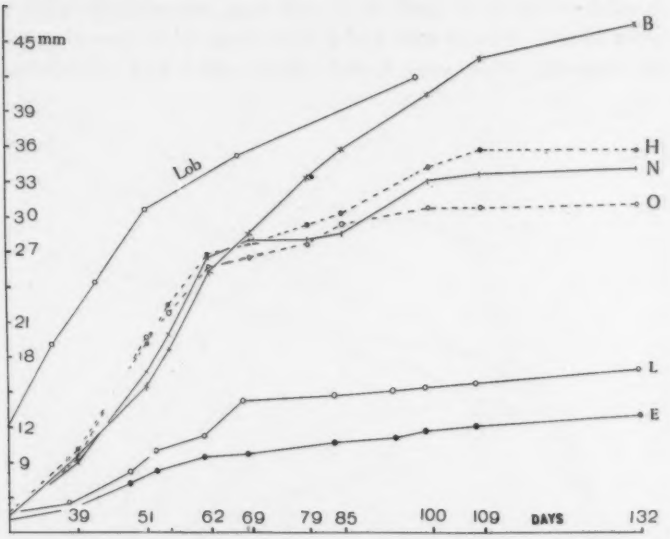


FIG. 6 shows effects of endocrin glands added to standard food (foul media?) and of distilled water on the mean length of snails hatched from the same mass of eggs and isolated at the age of 27 days. Light, temperature and volume of medium were the same except that two of *B* and 2 of *H* were kept in such broad flat vessels that the medium was less than one inch deep. All were fed leaf lettuce in quantity and kept in tap water, except *E* and *L*. *H*, *N*, and *O* were also given desiccated glands, mixed with wheat flour to make a dough and dried, two to three times each week. *B*, controle, 3 individuals. *E*, 3 individuals, distilled water, aerated 15–30 minutes daily, no sunlight, leaf lettuce and filter paper (Fig. 5, *N*). *H*, 3 individuals, anterior lobe of hypophysis. *N*, 2 individuals, thymus. *O*, 3 individuals, thyroid. *L*, 3 individuals treated as in *E*, except, sunlight through window daily (Fig. 5, *L*). *Lob*, master control (Fig. 1, *O*).

Twenty-four individuals hatched and were kept in a finger-bowl until they were 99 days old when the three largest (20, 15 and 10 mm. long) and the two smallest (7 and 5.50 mm. long) were changed to standard isolation cultures (Fig. 3, *H*) and within 60 days almost caught up with six snails which were isolated before hatching and fed leaf lettuce and filter paper (Fig. 3, *N*).

A mass of eggs was allowed to hatch and the 51 surviving young to remain in a fingerbowl until 70 days old when the two smallest individuals (Fig. 8, *S*) and the two largest individuals (Fig. 8, *C*) were isolated and reared under standard condition. Another mass of eggs was allowed to hatch and the 33 young to remain in a fingerbowl until 61 days old when the two smallest (Fig. 8, *H*) and the two largest (Fig. 8, *O*) were isolated and reared under standard conditions. Each mass culture was fed and water changed three to six times a week. The most significant points in the last two experiments are that even the

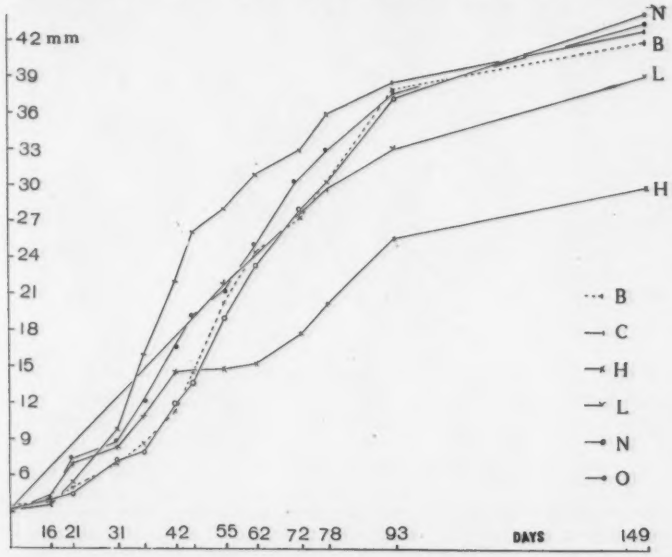


FIG. 7 shows effects of different food and media on snails all from a single mass of eggs, isolated when one week old (except that *L* is a mass culture) fed, excrement syphoned off two or three times each week and water poured out when foul, except *H*. All other conditions were the same. The mean length and age in days are given. *B*, 3 snails, one died before 71st day; iceberg lettuce and filter paper and occasionally boiled wheat. *C*, 3 snails, one died before 71st day, another died before last measurement, iceberg and leaf lettuce, dry elm leaves, apple peel and blotting paper. *H*, 3 snails, one died before last measurement; excrement not removed. *L*, mass control, 6 snails reared in a battery jar containing approximately 1500 ml. of tap water; leaf lettuce, boiled wheat and filter paper. *N*, isolation control, 3 snails; leaf lettuce boiled wheat and filter paper. *O*, 3 snails; same conditions as *H*, except that each was aerated 15–45 minutes every 2–3 days. All individuals are from one egg mass.

largest individuals were much stunted, that both the smallest and the largest individuals responded to the standard isolation treatment and that the worst stunted individuals grew more rapidly than the largest individuals. Since this snail reproduces by self-fertilization (Crabb, 1927*b*) individuals obtained from the same egg mass would be expected to have very similar genetic constitutions. Therefore, one would attribute the common occurrence of a few "dwarfs" and one to three "giants" in a

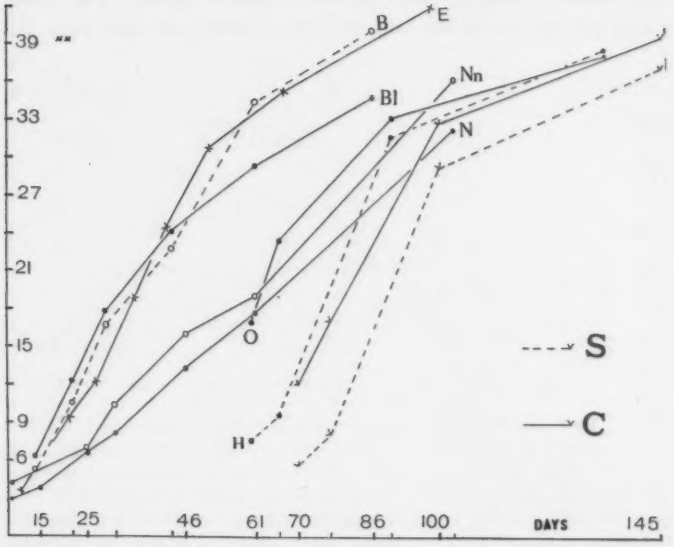


FIG. 8 shows effect of light, medium, crowding and food. The mean length only is given in each case. *B*, 3 snails reared in isolation from hatching, tap water, leaf lettuce, boiled wheat and filter paper; food and water changed 3 times each week; direct sunlight 1 to 3 hours daily. *Bl*, 6 snails treated as in *B*, except that excrement was not removed. *C*, see *S* and *C*. *E*, master control of 15 individuals (Fig. 1, *O*). *H*, 2 "dwarfs" and *O*, 2 "giants" of a mass culture of 33 individuals all hatched from the same egg mass and reared in a fingerbowl until 61 days old when *H* and *O* were isolated in fingerbowls and reared under standard conditions. *N*, 4 individuals, isolated in tap water in fingerbowls, fed iceberg lettuce, filter paper and occasionally boiled wheat and kept in total darkness at about 18° C. Food and water changed at night 2–3 times each week. *Nn*, mean length of 2 individuals (only one in last measurement) conditions the same as *N*, except that food was leaf lettuce, boiled wheat and dry elm leaves. *O*, see *H*. *S*, 2 "dwarfs" and *C*, 2 "giants" of a mass culture of 51 individuals, all hatched from the same egg mass and reared in a fingerbowl until 70 days old, when *S* and *C* were isolated in fingerbowls and reared under standard conditions.

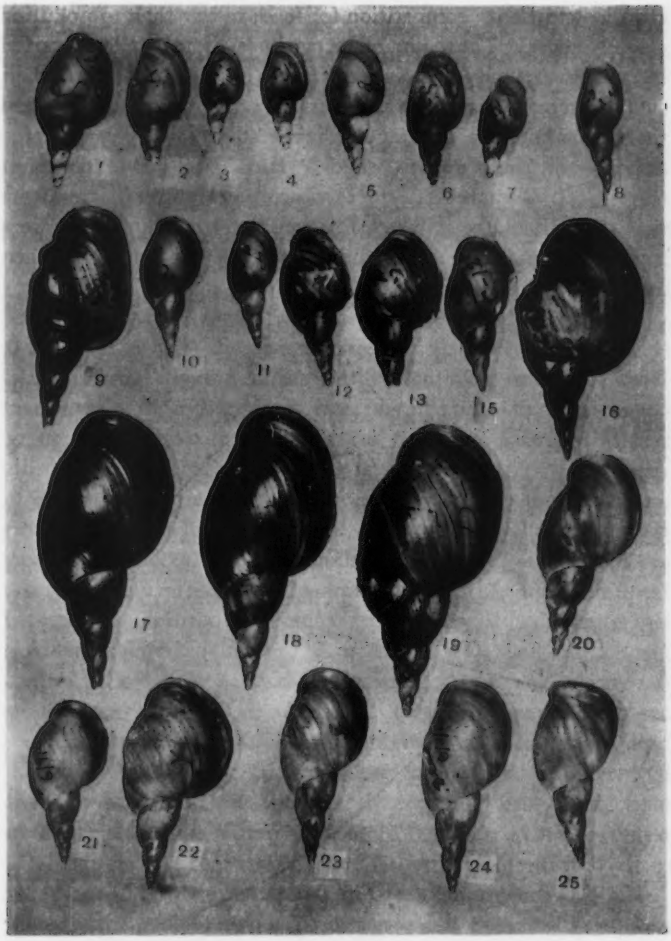


FIG. 9 shows the effect of favorable (Nos. 17–19) and unfavorable culture media on growth; food and other factors being equal. (× 1.) Nos. 1–7, distilled water and leaf lettuce (see Figs. 5, 6, *L*, *E*); ages at death were as follows; No. 1, 231 days; 2, 235; 3, 199; 4, 175; 5, 168; 6, 269; 7, 196. Nos. 1 and 2 are from group *B*, Fig. 5; Nos. 3 and 4, *H*, Fig. 5; 5 and 6, *L*, Figs. 5 and 6; 7, *N* Fig. 5 or *E*, Fig. 6. No. 8 (Fig. 7, *B*), tap water and iceberg lettuce, age 74 days. Nos. 9–16, foul media (Fig. 1, *N*) ages: No. 9, 306 days; 10, 80; 11, 97; 12, 165; 13, 168; 15, 70; 16, 280; No. 17 age 280 days; 18, 183 (from master control, Fig. 1, *O*). Nos. 19–25, ductless gland experiment (Fig. 6, *B*, *H*, *N*, *O*); No. 19, control (*B*), age 158 days; 20 (*N*), 196; 21 (*O*), 135; 22 (*O*), 165; 23 (*O*), 151; 24 (*N*), 135; 25 (*H*), 151.

crowded culture to competition for food, rather than to pollution of the media. The common occurrence of markedly dwarfed individuals reared in isolation (Fig. 1, *C*, *H*, *Nn*) and their sudden increase in growth at advanced ages (Fig. 1, *C*, *H*) makes the explanation of the factors involved in dwarfing and the response of the "dwarfs" and "giants" to good treatment (Fig. 8, *H*, *S*, and *O*, *C*) more obscure. However, it appears that the critical age at which this snail may be transferred from a crowded mass culture to standard isolation conditions and then attain normal adult size is near 150 days.

I have ascribed the retarded growth and highly convoluted shells of the eight snails fed endocrine glands (Fig. 6, *H*, *N*, *O*; 9, Nos. 20–25) to pollution of the media brought about by decomposition of gland tissue and flour rather than to direct results following ingestion of the gland mixture. It is to be regretted that these results are based upon so few experiments and that flour controls were not raised. The relation of age to the amount of shell distortion indicates that if pollution factors are responsible for the dwarfed, convoluted shells, those factors resulting from the decomposition of excrement (Fig. 1, *N*) are less potent than those resulting from decomposition of desiccated glands and flour (Fig. 6) as may be seen by comparing Nos. 9–16 with Nos. 20–25, particularly No. 12 with No. 22, which are the same age, in Fig. 9. Since for the last two years I have been successful in maintaining stock animals in crowded culture by keeping the aquaria well supplied with *Daphnia*, which markedly retard pollution, it appears probable that different results might be obtained by feeding certain endocrine glands to snails kept in much larger volumes of *Daphnia* stocked media.

The experiments described in this paper show that markedly retarded growth in the presence of adequate food is normally accompanied by pollution in any reasonable volume of media. Thus, after all, in the growth of this snail, pronounced effects of crowding are chiefly expressions of foul media.

## Conclusions.

1. A method of rearing pond snails in the laboratory for experimental purposes is described.

2. A diet composed of lettuce, boiled wheat grains and filter paper produced the best results in growth and in number and viability of eggs laid, provided the medium was favorable.

3. Food insufficiency and foul media are the most common growth inhibiting factors in snails reared in otherwise favorable media.

4. Extreme crowding markedly retards growth, but the individuals rapidly reach the norm after being isolated under standard conditions unless too old at this time, when they may fail to reach normal adult size.

5. Volume of medium appears to have relatively little effect on the growth of isolated snails, provided foulness is not permitted.

6. *Daphnia* introduced into the media markedly retard fouling in stock cultures.

7. Aëration promotes growth through reducing foulness of medium, by oxidizing decaying substances, but has no appreciable respiratory significance, since these animals normally breathe atmospheric oxygen.

8. Direct sunlight does not appear to increase the growth-rate over that of sunlight which has passed through plate glass.

9. There is no evidence that dwarfing produced by unfavorable culture conditions is transmitted.

## LITERATURE CITED.

**Baker, F. C.**

'11 The Lymnæidæ of North and Middle America, Recent and Fossil. Chicago Acad. Nat. Sci., Special Pub. No. 3.

**Coker, R. E., A. F. Shira, H. W. Clark and A. D. Howard.**

'22 Natural History and Propagation of Fresh-water Mussels. Bull. U. S. Bur. Fisheries, Vol. 37 (Document 893): 75–182.

**Colton, Harold S.**

'12 *Lymnæa columella* and Self-Fertilization. Proc. Acad. Nat. Sci., Philadelphia, 64: 173–82.

**Crabb, Ed. D.**

'27*a* Genetic Experiments with Pond Snails, *Lymnæa* and *Physa*. Amer. Nat., 61: 54–67.

**Crabb, Ed. D.**

'27*b* The Fertilization Process in the Snail, *Lymnæa stagnalis appressa* Say. BIOL. BULL., 53: 67–109.

**Diver, C. C.**

'25 Inheritance of Inverse Symmetry in *Limnæa peregra*. Jour. Genetics, 15: 113–200.

**De Vorges, Domet.**

'03 Note sur l'utilisation des sels calcaires des l'eau par les Mollusques. Bull. Soc. Zoöl. France, 28: 149–150.

**Ellis, Arthur Erskine.**

'26 British Snails. A Guide to the Non-marine Gastropoda of Great Britain and Ireland, Pliocene to Recent. Clarendon Press.

**Greenleaf, W. E.**

'26 The Influence of Culture Medium and Cell Proximity on the Rate of Reproduction of Infusoria. Jour. Exp. Zoöl., 46: 143–167.

**Hoffmann, H.**

'27 Physiologie. C. Wachstum. Bronns Klassen u. Ordnungen des Tier-Reichs. 3 (Buch 3): 1101–1108.

**Hogg, Jabez.**

'54 Observations on the Development and Growth of the Water-snail (*Limneus stagnalis*). Trans. Microsc. Soc. of London in Quart. Jour. Microscop. Sci., N.S., 2: 91–103.

**Hurst, Clarence T.**

'27 Structural and Functional Changes Produced in the Gastropod Mollusk, *Physa occidentalis*, in the Case of Parasitism by the Larvæ of *Echinostome Revolutum*. Univ. of Calif. Pub. Zoöl., 29 (14): 321–404.

**Isely, Frederick B.**

'14 Experimental Study of the Growth and Migration of Fresh-water Mussels. Report U. S. Commissioner of Fisheries, 1913 (Appendix III).

**Kulagin, N.**

'99 Zur Biologie der Infusorien. Le Physiologiste russe, 1.

**Legendre, R.**

'07 Sur un facteur important du nonisme expérimental: les excrétas. C. R. Assoc. Française p. l'avance. d. Sciences, 36e Session (Reims, part 2): 607–610.

**Legendre, R.**

'08 Recherches sur le nanisme expérimental. Influence des excreta. Arch. de Zoöl. Exp. et Gen., 4 Ser. 8 (Notes et Revue, No. 3), pp. lxxcii–lxxxiv.

**Mozley, Alan.**

'28 The Variation of *Lymnæa traskii* Tryon in Pond and Lake Habitats. Amer. Nat., 62: 286–288.

**Popovici-Baznosanu, A.**

'21 L'influence de quelques facteurs sur l'accroissement des gastéropodes d'eau douce. Arch. Zoöl. Exper., 60: 501–521.

**Robertson, T. B.**

'23 The Chemical Basis of Growth and Senescence. J. B. Lippincott Co.

**Semper, Carl.**

'74 Ueber die Wachsthums-Bedingungen des *Lymnæus stagnalis*. Arbeiten aus dem zoologisch-zootomischen Institut im Wurzburg, 1: 137–167.

**de Varigny, Henri.**

'94 Recherches sur le nanisme expérimental, contribution a l'etude de l'influence du milieu sur les organismes. Jour. d'Anatomie et Physiol. (30e ann.): 147–188.

**Vernon, H. M.**

'95 The Effect of Environment on the Development of Echinoderm larvæ, an Experimental Inquiry into the Causes of Variations. Philos. Trans., 186 (B): 577–632.

**Vernon, H. M.**

'99 The Relations Between Marine Animal and Vegetable Life. Mittheilungen a.d. Zool. Stat. z. Neopel 13: 341–425.

**Vernon, H. M.**

'03 Variation in Animals and Plants. Henry Holt & Co., New York.

**Warren, Ernest.**

'00 On the Reaction of Daphnia magna (Straus) to Certain Changes in its Environment. Quar. Jour. Microsc. Sci., 43: 199–244.

**Weymouth, F. W., H. C. McMillin and H. B. Holmes.**

'26 Growth and Age at Maturity of the Pacific Razor Clam *Siliqua patula* (Dixon). Bull. U. S. Bur. Fisheries (Document No. 984): 201–236.

**Whitefield, R. P.**

'82 Description of *Lymnæa* (*Bulimnæa*) *megasoma* Say, with an Account of Changes Produced in the Offspring by Unfavorable Conditions of Life. Bull. Amer. Mus. Nat. Hist. 1 (2): 29–37.

**Willem, Victor.**

'96 Observations sur la respiration cutanée des Limnees et son influence sur leur croissance. Bull. de l'Acad. Roy. des Sciences, des Beaux-Arts de Belgique, 66me Année, 3me Sér., 32: 563–577.

**Woodruff, L. L.**

'11 The Effect of Excretion Products of *Paramecium* on its Rate of Reproduction. Jr. Exp. Zoöl., 10: 557–581.

**Woodruff, L. L.**

'13 The Effect of Excretion Products of Infusoria on the Same and on Different Species, with Special Reference to the Protozoan Sequence in Infusions. Jour. Exp. Zoöl., 14: 575–582.

**Yung, Emile.**

'85 De l'influence des variations du Millieu physico-chemique sur le development des animaux. Arch. d. Sci. Phys. et Nat., 14: 502–522.

# PERMEABILITY DIFFERENCES BETWEEN NUCLEAR AND CYTOPLASMIC SURFACES IN *AMŒBA DUBIA*.

YUN-ICHI MORITA AND ROBERT CHAMBERS,

LABORATORY OF CELLULAR BIOLOGY, DEPARTMENT OF ANATOMY, CORNELL UNIVERSITY MEDICAL COLLEGE, NEW YORK CITY.

Fresh water amebæ readily stain with the pH indicator, methyl red (1). Not only is the hyaline cytoplasm colored yellow with the dye but also the nucleus, the contractile vacuole and the various cytoplasmic inclusions. This occurs within a few minutes in a concentration prepared by adding 1 cc. of the standard 0.4 per cent. Clark and Lubs solution of the dye (Hynson, Westcott and Dunning) to about 8 cc. of the culture water. In this mixture the amebæ survive for over twenty-four hours with no appearance of being injured.

The fact that the yellow color is the color of the alkaline range of the dye [1] suggested the possibility of testing the penetrability of hydrochloric acid into the nucleus from the cytoplasm of the ameba. It is already known that the living cell-membrane is impermeable to hydrochloric acid but it is not known whether the acid, when once introduced into the cytoplasm, will pass through the surface membrane of the nucleus.

The various structural components of amebæ immersed in a solution of methyl red take up the dye at different rates. The hyaline cytoplasmic matrix is the first to color yellow, then the nucleus, and finally the contractile vacuole and the food vacuoles. The food vacuoles, however, always remain distinctly paler than the contractile vacuole.

## EXPERIMENTAL RESULTS.

### 1. *Amebæ Stained with Methyl Red and Immersed in Solutions of Hydrochloric Acid.*

Amebæ, stained yellow with methyl red and then immersed in concentrations of N/1,600 and *N*/3,200 of HCl, usually became

[1] An aqueous solution of methyl red is yellow in its alkaline and red in its acid range. The turning point of the color is at the pH of about 5.5.

injured within 2–5 minutes. The injury was made apparent by a sudden change in color from yellow to red of a localized area which was quickly pinched off. Every few minutes this process was repeated so that the healthy remnant of the Ameba became successively smaller until the entire ameba was killed. In a moving ameba the first part to be injured was the tip of an extending pseudopodium. This phenomenon was best observed when the amebæ are placed in concentrations of $N/400$ to $N/800$ HCl, where the injury usually manifested itself within a couple of minutes, and in amebæ which put out large pseudopodia. In such cases there was a rapid flow of the yellow protoplasm to the tip of the pseudopodium where a small red spot suddenly appeared. The red color increased in extent as the injury effect spread. Usually the injured area is pinched off before the entire ameba becomes involved.

In strong solutions of the acid, *e.g.*, $N/200$, the injury quickly spreads from several spots on the surface of the ameba with the result that the entire ameba is killed before any pinching-off process can occur.

2. *Injection of Hydrochloric Acid into Stained Amebæ.*

An amount of approximately the volume of the nucleus of $N/200$ HCl or stronger was injected into the cytoplasm in the vicinity of the colored nucleus. The nucleus and the cytoplasm, about the site of the injection, immediately changed in color from yellow to red and the entire red mass, consisting of the injured cytoplasm and the nucleus, were then pinched off and discarded by the ameba.

With a more dilute concentration of HCl ($N/400$) the effect is not so drastic so that a reversible reaction could frequently be obtained. A small amount of $N/400$ HCl was micro-injected into a stained ameba, the tip of the micropipet being directed toward the nucleus at a distance of about half the diameter of the nucleus. Immediately after the injection the cytoplasm about the tip of the pipet turned red, after which the color of the nucleus likewise became red. The yellow color of the nucleus changed to red in a wave which spread from the border near the site of the injection. Within one or two seconds the red

color of both cytoplasm and nucleus returned to the original yellow. The reversion in color always occurred more rapidly in the nucleus than in the cytoplasm. The change in color of the nucleus from yellow to red and back to yellow was unaccompanied by any appreciable morphological change such as occurs when the nucleus is irreversibly injured.

Amebæ, in which the reversible reaction had been noted, were kept under observation for several days during which time they appeared to be quite normal.

### 3. *Injection of the Acid into Stained Amebæ in the Vicinity of the Contractile Vacuole.*

Injection of hydrochloric acid in the vicinity of the contractile vacuole produced no change in color of the interior of the vacuole unless the concentration of the acid is enough to cause irreversible injury. This is in marked contrast to the ease with which the nucleus changes in color and suggests that the membrane of the contractile vacuole, in this one respect, differs from that of the nucleus but is similar to that on the surface of the ameba. An alternative to this suggestion is that the HCl does enter but that the content of the vacuole is too highly buffered to be affected by the acid. However, the results of recent work (2) indicate that the concentration of dissolved substances in the vacuole is fairly low and that its content is mainly water.

## SUMMARY.

### 1. *Immersion Experiments.*

Amebæ, stained yellow with methyl red and immersed in solutions of HCl, exhibit injury effects by a sudden change in color from yellow to red in localized regions on the periphery. These regions are pinched off and discarded. In a moving ameba this injury first occurs at the tip of the extending pseudopodia.

### 2. *Injection Experiments.*

Sublethel concentrations of HCl which do not penetrate the ameba from without, will, when injected into the cytoplasm, readily diffuse into the nucleus without causing irreversible injury. On the other hand, the wall of the contractile vacuole

appears to be similar to that of the plasmalemma of the ameba in regard to the nonpenetrability of HCl.

## BIBLIOGRAPHY.

1. **Chambers, R.**

'28 Intracellular Hydrion Concentration Studies. I. The Relation of the Environment to the pH of Protoplasm and of its Inclusion Bodies. BIOL. BULL. 55,369.

2. **Howland, R. B., and Pollack, H.**

'27 Micrurgical Studies on the Contractile Vacuole. I., II. J. Exp. Zoöl., 48, 441.

# THE CULTURE OF *AMŒBA PROTEUS* IN A KNOWN SALT SOLUTION.

D. L. HOPKINS[1] AND PERCY L. JOHNSON.

*(Contribution from the Zoölogical Laboratory of The Johns Hopkins University,[2] October, 1928.)*

## INTRODUCTION.

Many methods [3] have been described for the culture of Amœba proteus; but in them very little attention has been directed to the salt composition of the media used. The method adhered to in this laboratory for several years is described by Edwards ('23). It consists of adding a few short pieces of timothy hay to spring or distilled water in finger bowls with subsequent inoculation with amœbæ and *Chilomonas*. Hopkins ('26), however, has shown that for certain types of physiological work this method is not satisfactory since the salt and H-ion concentrations do not remain constant. To obviate these difficulties he raised amœbæ in a modification of Ringer solution supplemented by a daily feeding schedule. Later he used another modification of Ringer solution which contained only the chlorides of calcium, sodium and potassium with a phosphate buffer, pH 6.6 (Hopkins, '28). In this solution it was found that the amœbæ could be raised only after a considerable period of adaptation. Amœbæ from a culture of any other composition placed into this solution, disappeared.

Aside from the adaptation process there was still another difficulty of equally as great importance. Hay contains considerable salt so that when it is added to cultures the inorganic compositions are altered in an unknown way. Roberts ('26) presents a salt analysis of timothy hay which will suffice to illustrate the salt composition (Table I.). The salt content of timothy hay is not always constant, varying from six to eight

[1] National Research Fellow.

[2] The authors are indebted to Professor S. O. Mast for helpful criticism and advise.

[3] Dawson ('28) gives a thorough review of this literature.

per cent. ash, depending upon the environmental conditions, *e.g.*, climate, altitude, etc.

TABLE I.

| Salts | Per Cent. Dry Weight. |
|---|---|
| Total ash | 7.70 |
| Silica free | 4.00 |
| $P_2O_5$ | 0.4712 |
| CaO | 0.3942 |
| $Fe_2O_3$ | 0.0393 |
| $Na_2O$ | 0.0023 |
| $K_2O$ | 2.375 |
| Cl | 0.239 |

It appears from this analysis of the inorganic composition that a considerable amount of physiologically active substances is introduced into amœbæ cultures with the hay, thus causing alterations in the composition of the culture media. An attempt was made, therefore, in the experiments described in the following pages, first, to remove the salts from the hay and then to adjust the salt composition favorably for the growth of amœbæ and yet to control the H-ion concentration.

## METHODS AND RESULTS.

Two methods were used to extract salts from timothy hay, *A* and *B*.

*A*. Since hay contains protein probably in the form of ampholytes, it is expected that it holds salts in combination which are not removed when extracted with distilled water. The procedure adopted by Loeb ('22) for extracting the salts from gelatin when the H-ion concentration has a value equal to the isoelectric point of the ampholyte, was applied to timothy hay. The method used follows: 75 gm. hay were ground in a meat chopper and extracted two times with 1.5 liters of distilled water. The hay was then added to 1.5 liters of distilled water and the mixture brought to a H-ion concentration of pH 8.0 by the addition of 60 cc. *M*/20 KOH, then the solution was filtered off. After washing with distilled water the hay extract had a H-ion concentration of pH 6.6. Following this, more distilled water was added and the H-ion concentration increased to pH 4.5 by the addition of 10 cc. *M*/5 HCl. The acid was then washed out until the filtrate had a H-ion con-

centration of pH 5.8. The hay was then dried at a temperature of 50°. After burning a sample of this hay it was found that only 1.1 per cent. of the total dry weight remained as ash.

*B.* In extracting the salts from the hay as described above, considerable organic substance was removed. In order to avoid this, another method was resorted to which afforded better results. This method of electro-dialysis was used by Livingston ('07) for separating electrolytes from non-electrolytes in manure extracts. The apparatus used is represented in Fig. 1. In this

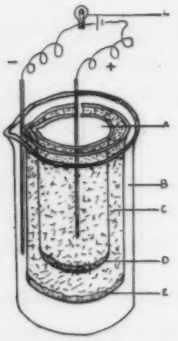


FIG. 1. Apparatus for dializing timothy hay. *A*, inner chamber containing distilled water into which is inserted the anode of the electric circuit; *B*, outer chamber containing distilled water into which is inserted the cathode; *C*, middle chamber containing finely ground hay; *D*, and *E*, collodion membranes (the collodion imbedded in bolting cloth for support); *L*, carbon lamp in the circuit to increase the resistance.

apparatus when the circuit [1] is closed the cations collect at the negative pole and the anions at the positive pole, leaving the undialyzable material behind. Frequently the acid and basic solutions formed at the two electrodes were replaced by distilled water. The dialysis was carried on for three days.[2] The hay was then dried in an oven at 50° C., after which a sample was tested and the ash content found to be 1.3 per cent. Since owing to the relative immobility of silicon ions it is very probable that 75 per cent. of the inorganic substances remaining in the

[1] A P. D. of 55 V. is best.

[2] Since it is difficult to prepare the collodion membranes, in the way used here, so that they will not leak, the use of diffusion shells is more practicable.

hay after dialysis consisted of silicon compounds and these are very inactive physiologically. This method of removing the salts appears to be very satisfactory.

This hay with very much reduced salt content was now used to culture amœbæ. We tried various mixtures containing different concentrations of the chlorides of calcium, sodium, potassium, and potassium phosphate buffer. After a number of attempts we found a mixture which on the first trial gave satisfactory results in all of fifteen cultures; the amœbæ on being placed in these cultures multiplied rapidly until at the end of two weeks each culture contained amœbæ in considerable numbers. The composition of this mixture is given in Table II.

TABLE II.

SOLUTION I.

| | | |
|---|---|---|
| $CaCl_2$ | .02219 gm. | .0002 M. |
| NaCl | .23380 gm. | .004 M. |
| KOH | .0499 gm. | .00088 M. |
| $KH_2PO_4$ | .34030 gm. | .0025 M. |
| Dialized hay extract | 10 cc. | |
| $H_2O$ | to 1,000 cc. | |

This mixture was ordinarily prepared by making a solution of the first two mentioned salts thirty times as concentrated, then taking 33.3 cc. of this and adding to it 50 cc. of Clark and Lubb's phosphate buffer 6.6 (with KOH instead of NaOH); to this was added 2 gm. of the dialyzed hay, 10 cc. of the resulting dialyzed hay extract, a liberal amount of centrifuged *Chilomonas* or better *Colpidium* grown on the same media and distilled water to make 1,000 cc. The cultures made up in finger bowls, 100 cc. solution to a dish, were then inoculated with *Amœba proteus*.

The original success with this medium seems to have been accidental, since subsequent attempts to culture amœbæ in the medium were not universally successful. If fresh cultures were inoculated with amœbæ from the original fifteen cultures they were as successful as the originals, but if inoculated with amœbæ from spring water cultures uninterrupted growth and reproduction occurred in a relatively few cases. Therefore, it was concluded that in the inoculation of the original fifteen cultures, amœbæ were used, which accidentally happened to be in the right physiological condition, perhaps the culture from which

they were obtained had nearly the same composition as the new medium.

For inoculation with amœbæ from ordinary dilute spring water cultures, the buffer in the medium of Table II. appears to be too concentrated. Therefore, in order to insure success with amœbæ from dilute cultures, it is necessary to reduce the amount of buffer considerably, inoculate with amœbæ, then gradually increase the amount of buffer until sufficient buffer is present to keep the H-ion concentration constant. To do this the buffer content of the medium was reduced to 5 cc. standard buffer per 1,000 cc. medium and inoculated with amœbæ from ordinary spring water cultures. In the medium with this reduced buffer content, a high degree of success was obtained, in over 70 per cent. of the cultures, the amœbæ on being placed in it continued to grow and multiply such that within two weeks great numbers of amœbæ were present in the cultures. Then 10 cc. of standard buffer was added per 1,000 cc. culture. This addition did not noticeably affect the rate of growth and multiplication. In this manner by adding the buffer gradually healthy cultures of amœbæ, rapidly dividing by fission, could always be obtained, which contained sufficient buffer to hold the H-ion concentration constant, namely, 50 cc. standard buffer per 1,000 cc. culture.

## Discussion.

In making up a 100 cc. culture with solution I., .2 gm. of hay is added. If this hay is undialyzed it contains approximately 8 per cent. salts or 0.016 gm. salts of an unknown nature. The weight of the known salts in 100 cc. of solution I. is 0.0647 gm. Therefore, if undialyzed hay is added, the unknown salt concentration of the culture is 19.9 per cent. of the total salts present. When .2 gm. of dialyzed hay, having from 1.0 to 1.4 per cent. inorganic material in it, is added in place of the undialyzed hay, there is introduced only from .002 to .0028 gm. unknown salts, *i.e.*, 2 to 3 per cent. As stated above since it is probable that the greater amount of the salts left in the dialyzed hay is silicon compounds, those that are left have little physiological effect.

SUMMARY.

A medium, the inorganic composition of which is accurately known, was devised, in which *Amœba proteus* grows and multiplies rapidly. By adding potassium phosphate buffer gradually, so as to allow time for adaptation on the part of the amœbæ a concentration of buffer may be added which is sufficient to hold the H-ion concentration constant and yet not interfere with the growth and multiplication of the amœbæ.

BIBLIOGRAPHY.

**Dawson, J. A.**

'28 The Culture of Large Free-living Amœbæ. Am. Nat., Vol. 62, pp. 453–466.

**Edwards, J. G.**

'23 The Effect of Chemicals on Locomotion in *Amœba*. I. Reactions to Localized Stimulation. Jour. Exp. Zoöl., Vol. 38, pp. 1–44.

**Hopkins, D. L.**

'26 The Effect of Hydrogen ion Concentration on Locomotion and Other Life-processes in *Amœba proteus*. Proc. Natl. Acad. Sci., Vol. 12, pp. 311–315.

**Livingston, B. E.**

'07 Further Studies on the Properties of Unproductive Soils. U. S. Dept. Ag. Bureau of Soils. Bull. No. 36, p. 51.

**Loeb, Jacques.**

'22 Proteins and the Theory of Collodial Behavior. First Edition, New York. 292 pp.

**Roberts, E. N.**

'26 Wyoming Forage Plants and their Chemical Composition. Studies No. 7, Univ. Wyoming Ag. Exp. Stat. Bull. No. 146, p. 35.